

The Effects of Adrenergic Antagonists on Spatial Memory in the Zebra Finch
(*taeniopygia guttata*)

by
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2014

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ACKNOWLEDGEMENTS

First and foremost, I cannot express enough thanks to my research advisor, Dr. Lainy Day, for her continued support and encouragement. I would also like to thank my committee, Dr. Richard Buchholz and Dr. Karen Sabol, for their guidance and willingness to work with me. I would like to recognize the Sally McDonnell Barksdale Honors College for providing me with the opportunity to become involved in this research.

My completion of this project would not have been possible without the support from the members of the Day Lab. I would like to thank Amy Hribar for providing the inspiration and foundation of this project. I would also like to thank J'undra Pegues and James Roberts for their constant help and support, both in and out of the lab. I owe my success to the Day Lab members, and for that I am thankful.

Finally, I would like to thank my friends and family. Without their continued encouragement, this would not have been possible. To everyone involved both physically and emotionally, I give my deepest gratitude.

ABSTRACT

TAYLOR ALEXANDRA WILLIAMS: Effects of Adrenergic Antagonists On Spatial Memory In

The Zebra Finch (*Taeniopygia guttata*)

(Under the direction of Dr. Lainy B. Day)

The adrenergic system appears to be involved in the consolidation and reconsolidation of hippocampally dependent spatial memories in mammals. Based on connectivity, cell types, ontogeny and receptor distribution, the avian hippocampus is thought to be a homolog to the mammalian hippocampus. The adrenergic system appears to be fairly conserved but may show some species specializations. To determine if the adrenergic system plays a role in spatial learning and memory in birds, we used a series of experiments to investigate the role of α - and β -adrenergic receptors on spatial navigation and memory in an avian species, zebra finches, using the Day Escape Maze, a dry maze analog of the Morris Water Maze. Experiment 1 investigated the role of the β -adrenergic receptor antagonist, propranolol (20 and 40 mg/kg) and saline on interference with reconsolidation and long term recall of spatial learning when drugs were given immediately after memory reactivation. Experiment 2 analyzed the role of propranolol in spatial memory when drug delivery was given at two time points after memory reactivation. Birds were injected with 60 mg/kg of propranolol or saline immediately following reactivation or 25 min after reactivation and spatial memory was

assessed using specific probe trials. Experiment 3 assessed the role of α -adrenergic receptors in spatial memory using a receptor antagonist, Phentolamine. Birds were injected with 45 mg/kg either immediately after memory reactivation or 25 min after reactivation. Collectively, these three studies suggest that, in contrast to mammals, neither propranolol nor Phentolamine given at various doses and time points after reactivation of spatial memory impairs spatial recall or spatial memory in the zebra finch. Thus, across vertebrate taxa, the effects of norepinephrine on spatial memory reconsolidation may not be conserved or the distribution of adrenergic receptors in the hippocampus may not be conserved.

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INTRODUCTION

In order for memories to be created, a particular set of neuro-processes must occur. According to the Multiple Trace Hypothesis, a given memory must be encoded in different ways at different times after a learning process. During exposure to a stimulus in which sensory channels are gathering information, short-term memories (STM) are created. This form of memory usually lasts only for seconds, or as long as the stimulus continues (Breedlove et al., 2007). Further neural processes can convert STM into intermediate-term memory (ITM). Consolidation of ITM into more permanent memories creates a long-term memory (LTM). LTMs are an enduring form of memory that can last days, months, or years and have a very large capacity (Breedlove et al., 2007). The *consolidation* of these memories infers that, after learning, memory is initially in a labile state, but becomes stable and resistant to changes over time (Alberini, 2011). The temporal boundaries between these phases can vary between species, tasks, and brain regions involved, but the appearance of these stages and the control of these stages by distinct physiological mechanisms are conserved among vertebrates and most invertebrates (see Fig.1).

Underlying neural-mechanisms facilitate memory consolidation. A process known as long-term potentiation (LTP) is suggested to play a role in consolidation of most types of memory. LTP is a stable and enduring increase in the strength of a synapse following repeated, strong stimulation of the presynaptic inputs (Breedlove et

al., 2007). Glutamatergic neurons involved in LTP contain AMPA and NMDA receptors, which are both sensitive to glutamate but in different ways and which have different functions in LTP. Moderate levels of stimulation by the neurotransmitter glutamate activate only the AMPA receptors. The NMDA receptors do not respond, because magnesium ions (Mg^{2+}) block the NMDA receptor's integral Ca^{2+} channel. Repeated activation of the AMPA receptors causes a rapid depolarization of the neuron, which in turn drives the Mg^{2+} out of the NMDA channel allowing Ca^{2+} into the cell, resulting in further depolarization. The influx of Ca^{2+} also activates kinases, which are enzymes that catalyze phosphorylation and regulate secondary signal cascades (Breedlove et al., 2007). These protein kinases induce LTP in two ways: first, they promote the movement of latent AMPA receptors from the interior of the neuron to the cell membrane which increases the sensitivity of the neuron: second, the kinases, such as protein kinase A (PKA), phosphorylate and activate the transcription factor cAMP responsive element binding protein (CREB). CREB binds to the promoter regions of many genes and regulates the transcription of those genes. The regulated genes produce proteins that evoke a retrograde signal from the postsynaptic neuron, which instructs the presynaptic neuron to release more neurotransmitter. The increase in both the number of postsynaptic receptors and the amount of neurotransmitter released makes the synapse more responsive to future stimulation from the same presynaptic pathways that initiated LTP. In addition, CREB may regulate genes that result in growth of dendritic

spines and increased number of dendritic spines on the post-synaptic cell, thereby increasing sensitivity. This means that less stimulation of the presynapse is now required to evoke the same level of post-synaptic response that required higher stimulation from a larger number of inputs before LTP. Hypothetically, this is why we can recall an entire event when exposed to only an element of the event. For example, the smell of fresh baked cookies can excite the entire neural network and evoke memories of making cookies at a young age with your grandmother in her kitchen. The increase in receptors on the postsynaptic neuron constitutes early-LTP, and it is hypothesized to regulate ITM. Drugs that inhibit calcium-calmodulin kinase (CaMK), a kinase involved in the addition of AMPA receptors to dendritic surfaces, interfere with the formation of ITM (Breedlove et al., 2007). Late-LTP involves synaptic growth and requires gene transcription and protein synthesis (Pang et al. 2004), and is believed to mediate LTM. Inhibitors of protein kinase C (PKC), a kinase involved in activating CREB, are shown to prevent the formation of LTM (Breedlove et al., 2007).

Until recently, the consolidation theory proposed that memories are stable once stored (Tronson, 2007), but recent studies have suggested otherwise. A study performed by Tronson and his colleagues used protein synthesis inhibitors (PSIs) to investigate the nature of a consolidated memory. They injected PSIs after the recall of a previously consolidated memory, which caused a disruption in the original memory. This suggested that consolidated memories that are recalled enter a labile period, and

require an active process to re-stabilize and maintain the memory for future retrieval (Tronson, 2007). This study, and many others like it, has led researchers to propose the “Reconsolidation Hypothesis,” which implies that every time a memory is reactivated, it must undergo a process of reconsolidation to be maintained (Alberini, 2005). The retrieval of this long-term memory into a labile phase followed by its reconsolidation is hypothesized to be a storage mechanism, strengthening the duration of the original memory (See Fig. 2; Tronson, 2007). Memory reactivation involves a certain set of memory processes similar to those in consolidation, but research suggests that reactivation and consolidation are two distinct processes (Crowe et al., 2008). Studies of the cellular mechanisms of reconsolidation indicate that the process involves a complex intracellular cascade, beginning with receptor activation and ending with protein synthesis (Crowe et al., 2008).

Behavioral evidence supports the idea that reconsolidation can involve altering memories. Reactivated memories are vulnerable, and they can be altered or eliminated (Nadel et al., 2012). New information provided at the time of recall can add new aspects to memory, so that later evocation of that memory is likely to reactivate newer traces along with older traces to produce a distorted, or “false” memory (Breedlove et al., 2007). The addition of new information to an existing memory can also increase the intensity of a response to stimuli. The attachment of non-related stimuli to the reconsolidated memory is the neurological basis of Post-Traumatic Stress Disorder

(PTSD) (Breedlove et al., 2007). PTSD is initiated by a stimulus that evokes a fear response. The memory is consistently reactivated, and new stimuli in the environment, when attached to the memory, become new triggers to reactivate that memory again (Donovan, 2010). PTSD studies also suggest that, apart from the external stimuli affecting the memory, internal cellular mechanisms could influence the consolidation and reconsolidation of that memory in part because of the emotional content of the memory which involves activation of the noradrenergic system.

Long-term potentiation, required for the consolidation and reconsolidation of memory, is influenced by noradrenergic systems. Norepinephrine (NE) is suggested to be an essential modulator of memory through its ability to regulate synaptic plasticity (Tully, 2010), a key component of LTP. NE is a catecholamine produced by dopamine β -hydroxylase and is released either as a hormone into the blood or a neurotransmitter into the brain (Tully, 2010). NE is primarily produced by the locus coeruleus and is carried by projections of the locus to many specific sites of release throughout the brain (Tully, 2010). NE binds to different alpha- and beta-adrenergic receptors (α -ARs & β -ARs, respectively) to perform specific functions. Adrenergic receptors (ARs) respond to systemically released NE, and each adrenergic receptor subtype mediates distinctive actions via modulation of various intracellular pathways (Gibbs et al., 2005). These different receptors play a role in STM, ITM, and LTM phases of memory. Kety (1970) proposed that activation of β -adrenergic receptors by NE could result in the facilitation

of synaptic transmission through increases in cAMP concentrations and new protein synthesis. (as cited in Tully, 2010). Recent studies have shown that NE-driven phosphorylation of a GluR1 (glutamate receptor type 1) subunit may facilitate latent AMPA receptor trafficking to synaptic sites during induction of LTP. One study testing the effects of NE on GluR1 exposed rats to fox urine, which increased NE in the brain (Hu et al., 2007). Analyzing the brain slices revealed increased phosphates on the GluR1 receptors and an increased ability of these receptors to be recruited to the synapse (Hu et al., 2007). Another study using rats suggests that NE increases synaptic plasticity through activation of β -ARs involved the cAMP/PKA pathway (Tully, 2010). NE is capable of affecting the synaptic plasticity in both consolidation and reconsolidation. For example, propranolol, a β -AR antagonist, can impair acquisition of spatial information in rats running a Y-maze, suggesting that NE is involved in modulating memory processes at the time of learning (Sun et al., 2011). Whereas, in military veterans with PTSD, propranolol decreased the association between fear stimuli and memory during reconsolidation (Donovan, 2010), implicating the role of NE in reconsolidation.

Memory formation is a complex process that requires different brain systems acting in concert, with the physiological events in one brain region affecting other brain regions (Gibbs et al., 2008). All areas in the brain that have LTP, NE supplied via locus coeruleus innervation, and contain adrenergic receptors are susceptible to NE related alterations in memory reconsolidation. There are numerous brain regions innervated by

the locus coeruleus, each area may contain various adrenergic receptor types, and various brain regions underlie different types of learning. There are many types of learning and memory that can be altered by NE system action on specific memory processes and stages of memory. For example, one study showed the distinct roles of NE and adrenergic receptor subtype on the modulation and consolidation of one-trial, discriminated, avoidance learning in the chick intermediate medial mesopallium (IMM – homologous to the mammalian brain cortex) and the medial striatum (MSt) of the basal ganglia (Gibbs et al., 2005). In the IMM, activation of β_3 -ARs, a sub-type of β -adrenergic receptors, is important for ITM activation, and β_2 -ARs are important for the consolidation of ITM into LTM. In the MSt, β_1 -ARs are important in STM, and are most likely involved in attention and arousal. Overall, this study suggests that based on the type of learning, NE acts in different brain regions at different times in memory processing, enhancing memory through distinct populations of ARs (Gibbs et al., 2005). Therefore the phases of memory affected by NE are strongly influenced by the AR distribution in the brain region involved. Not only are the conversions between STM, ITM and LTM affected by NE and brain region, but the specific types of memory processes are also affected by specific receptor sub-types in different brain regions. Different brain regions are responsible for processing particular types of learning and their memory, such as fear conditioning, spatial learning and procedural learning. The likelihood of memories being susceptible to alteration by NE is based on the AR

distribution and sub-type in the specific brain region responsible for that type of memory. For example, spatial memory is controlled by the hippocampus, and it is known that β -adrenergic receptors are found in the mammalian hippocampus (Duncan et al., 1991). Spatial memory for escape platform location in a Morris Water Maze is inhibited when the β -adrenergic receptor antagonist propranolol is administered following recall of the memory (Cahill, 2008). These studies show how the role of NE in memory consolidation is dependent on AR distribution in the critical brain regions.

As stated, the role of NE is dependent on the type of AR and the distribution of that type of AR in the hippocampus. In birds, at least in chicks and zebra finches, the α - and β -AR distribution in certain areas of the brain are known. The song system of zebra finch has α -ARs (Velho et al., 2012) and β -ARs have been found in the chick hippocampus (Gibbs et al., 2008), but the distribution of ARs in the zebra finch hippocampus have yet to be determined. However, because hippocampal morphology is conserved among birds and mammals (Mayer et al., 2012), it is reasonable to suggest there is conservation of AR type in the hippocampus of birds and mammals and that NE would act on reconsolidation of spatial memories in birds the same way it does in rats. To test this, we used a spatial navigation paradigm to assess the role of NE in reconsolidating memory following recall of spatial memory in birds. Because it is not clear whether α - or β -ARs are present in the hippocampus of our model species, both an α -AR antagonist (Phentolamine) and a β -AR antagonist (propranolol) were administered

following the reactivation of spatial memory in zebra finches, and the ability of the birds to perform in a manner consistent with training was analyzed.

GENERAL METHODS

Subjects

For all experiments, we used adult female zebra finches (*Taeniopygia guttata*) of similar age bred in an aviary of the University of Mississippi. Females were housed in a same-sex aviary upon sexual maturation in a room kept on a 13-hour light schedule, with food and water available continuously except during training trials. Twenty-four hours prior to experimentation, the females were placed into cages (length 60.9cm, width 40.6cm, height 40.6 cm) for the duration of training and testing.

Apparatus

The Day Escape Maze (Fig. 3) was used in all experiments. An aviary (length 148.6cm, width 71.1cm, height 188.2cm) was lined with black cloth, so that no external light or objects could be seen from the inside. Four light bulbs were positioned in the upper corners of the aviary. Four distal cues were positioned on the aviary walls that we designated as north, south, east and west. The cues included a yellow star, a purple triangle, a pink oval, and a green cross on the south, east, north, and west walls of the aviary respectively. These cues were equidistant from the floor and ceiling of the aviary, and centered on the side in which they were placed. Two perches extend the width of the aviary about 25 centimeters below the ceiling and on opposite ends equidistant from the walls. A camera (ImagingSource) was secured to the top of the aviary near the

north wall of the aviary, focusing down on the arena below. To line the arena up with where the camera was attached, the arena was not centered in the middle of the aviary. It was 48.3cm from the back wall opposite the door and 20.32cm from each side wall. The escape maze is a clear cylinder (30cm in diameter and 15.2cm tall), made from extruded Plexiglas with a 5.4cm escape hole cut 7cm above the hotplate and a clear Plexiglas lid. The floor of the maze is a ceramic tile heated by an electronic hot plate. The hotplate was maintained at $\sim 50^{\circ}\text{C}$. This is sufficient to motivate the finches without causing severe stress or tissue damage. The cylinder is seated on a metal stool, bringing it closer to the camera. The escape hole was positioned in the Northwest quadrant of the cylinder according to the cardinal designations of the aviary walls. This assures that the finch will not be able to determine the position of the escape hole by simple approach or avoidance of a single cue.

EXPERIMENT 1

Studies have shown β -AR antagonists inhibit passive avoidance memory consolidation in the avian hippocampus (Gibbs et al., 2008). In this experiment, we were looking for a dose effect of propranolol on hippocampal spatial memory reconsolidation in zebra finches. One study showed that 60 mg/kg of propranolol led to undesirable side effects, such as sedation and trembling (Velho et al., 2012). Therefore, we decided to inject the birds with 20 mg/kg and 40 mg/kg propranolol dissolved in 1mL to a concentration of 20mg/ml or 40mg/ml and then given as 1ul/g of bird.

As far as we are aware, no published research has examined the role of β -AR in spatial learning of birds, nor studied how β -AR antagonists affect spatial memory. Studies performed in rats show that spatial memory is inhibited by the β -AR antagonist propranolol given immediately following a reactivation trial (Cahill et al., 2000). Another study performed on chicks showed that β -AR antagonists inhibited the memory of color discrimination in the hippocampus (Gibbs et al., 2008). Based on the mammalian model of spatial memory, and the evidence from avian species that β -ARs are important for memory reconsolidation, one would predict that β -AR antagonists would hinder reconsolidation of a spatial memory in birds.

Method

Subjects

Just prior to the experiment, 24 female zebra finches were selected at random, weighed, and housed in individual carrying cages (length 31.1cm, width 15.9cm, height 15.9cm) for easier access to birds during experimentation.

Behavioral Testing

Birds were divided into 2 batches of 12 birds with the morning batch tested starting at 11:00 a.m., and the afternoon birds starting testing at 3:00 p.m. Timing was consistent throughout the experiment. To start the experiment, birds were taken into the testing room. In order to keep the birds in a more relaxed and manageable state, the lights in the testing room remained off, and the experimenter used flashlights to conduct the experiment. The birds were not capable of seeing inside the aviary containing the Day Escape Maze. One at a time, each bird was removed from the individual carrying cage. The experimenter then entered through the door of the aviary. The lights inside the aviary remained off, preventing the birds from seeing any cues before behavioral testing started. The experimenter opened the lid of the escape maze and placed the bird inside. The placement of the birds inside the maze was randomized throughout the experiment between the north, east, south and west quadrants, but was the same for each bird on a particular testing trial which began immediately upon closing the lid. At this time, the experimenter quickly and carefully exited the maze, turned on the lights inside the aviary and started automated tracking software Ethovision™ (Noldus Information Technology, Virginia) and a stopwatch used for

backup time data in case of software failure. All birds could be viewed on a computer monitor during performance, and the software could be manually stopped in cases where the bird perched on the escape hole instead of exiting – obviously knowing the location of the hole but not moving into the aviary to trigger the software to stop tracking and end the trial. Each bird was allotted 2 minutes to escape. Once the bird escaped, the stopwatch was stopped, the time was recorded, and the tracking software stopped when the bird exited the arena. If the bird failed to escape within 2 minutes, the bird's latency was recorded as 120 seconds, and the experimenter entered the aviary and gently guided the bird to the escape hole. After escaping the maze, the bird was given a 60 second resting period in the aviary during which birds typically alighted to the perches provided. This resting period should provide positive reinforcement for exiting the maze. EthovisionTM captured the bird's movement, and three dependent measures were collected: escape latency, distance traveled, and traveling velocity. After completion of the first trial, the bird was returned to its isolation cage, and the next bird was run, resulting in an intertrial interval of about 30 minutes. Each bird completed 3 more trials, being placed into each of the 4 quadrants of the maze indicated by the cardinal designations and given a total of 4 trials per day. Both batches of birds trained for 4 consecutive days, resulting in 16 trials total per bird.

Probe Trial

On the 4th day, following the completion of the 4th trial of the day, a probe trial was conducted in order to determine if the birds had learned the spatial location of the escape hole. The cylinder used for the training days was replaced with an identical cylinder that did not have an escape hole. For this specific experiment, the stool and the hotplate were rotated 180°. In reference to the cues on the wall of the maze, the escape location was now in the Southeast quadrant. The purpose of the rotation of the of the stool was to ensure the birds were not using proximal cues on the hotplate, but that they were using the distal cues positioned on the walls of the aviary. While the hotplate temperature dial is just barely visible, it is possible that this proximal cue was being used to guide escape, and this probe trial would identify if this were the case. For the probe trial, the bird was placed in the maze, were its movements and behavior were recorded for 120 seconds. As in training trials, we used Ethovision software to collect the dependent measures of latency, distance and velocity in each quadrant of the maze. If spatial learning of the distal cues has occurred, the bird will spend most of its time in the northwest quadrant, were the escape hole was originally indicated by the distal cues before the rotation of the hotplate.

Reactivation, Injection and Recall

On the 5th day of the experiment, the 24 finches were randomly divided into 3 groups: saline 0.9%, 20mg/kg of propranolol, and 40 mg/kg of propranolol. Prior to drug injection, each bird was given a single reactivation trial, using the original apparatus and

set up with the escape hole in the original NW quadrant. Reactivation trials were used to instantiate re-consolidation of memory, shortly after which administration of adrenergic antagonists are predicted to interfere with spatial memory. An intracoelomic (IC) injection was administered 5 minutes after the reactivation trial. The finches were then tested for recall of the spatial location of the escape by examining time to escape using the original escape cylinder 24 hours post-injection, 72 hours post-injection, and one-week post-injection.

Increased Dosages

Initial observations of the birds' performance suggested that memory was maintained post-injection of 20 and 40 mg/kg doses. Thus, we decided to administer a higher dose of propranolol (80 mg/kg) to the birds after recall at 1 week. The birds were given 6 more days of training, consisting of 1 trial per day. On the 7th day, 1 week after their first injection, they were given another reactivation trial followed by an injection 5 minutes after the reactivation trial. The first 2 birds injected showed postural and sympathetic side effects after this high dosage of propranolol, thus no other birds were injected. Despite the side effects, the birds recovered over several hours and their memory recall was tested 24 hours and 1 week post their second injection. All memory recollection trials were conducted as for the lower dosages and were recorded in EthovisionTM to collect the three dependent measures of distance, latency and velocity.

Data Analysis

To confirm maze learning prior to reactivation and drug treatment, we performed a one-way repeated measures ANOVA on probe x cued and un-cued quadrants. The cued quadrant was the one indicated by the distal cues, the NW quadrant, and we used the average of the other three un-cued quadrants. This test gives us a direct comparison between the time spent in the quadrant indicated by the distal spatial cues versus time spent in all the other quadrants. It is used when the data suggesting a bias to one quadrant is strong enough that it is not necessary to compare all quadrants to each other using planned comparisons. Significant main effects in ANOVAS were followed by post-hoc t-tests using sequential Bonferroni correction. Using repeated measures ANOVA for trial x treatment, the dependent measures (distance, latency, and velocity) of the reactivation trial, and trials given 24h, 7h, and 1 week post-injection were examined for differences in maze performance pre and post-treatment.

Results and Discussion

To examine the data for meeting the assumption test of the ANOVA, we ran tests of sphericity. Tests of sphericity were significant for latency ($\chi^2(5)=100.41$, $P<0.0001$) and velocity ($\chi^2(5)=19.567$, $P=0.002$), but not significant for distance ($\chi^2(5)=4.609$, $P=0.47$). We thus used the Greenhouse-Geisser correction factor to examine the significance for within-subject effects for the dependent measures. There was no significant difference for the main effect of treatment, nor any trial x treatment effects for distance [Treatment: $F(2,21)=1.194$, $P=0.32$; Treatment by trial:

$F(2.578, 54.144)=0.765$, $P=0.58$], latency [Treatment: $F(2, 21)=0.704$, $P=0.51$; Treatment by trial: $F(2.232, 23.438)=0.972$, $P=0.40$], and velocity [Treatment: $F(2, 21)=0.940$, $P=0.41$; Treatment by trial: $F(3.830, 40.218)=3.001$, $P=0.054$] between the reactivation trial and the recall trials at 24h, 72h, and 1 week (Fig.4). Velocity was examined as a control to make sure the birds were still capable of full motor ability and flight speed after drug treatment.

We used the pre-treatment probe trial as a way to indicate learning of the task and since this probe occurred before any manipulation of the subjects, we predicted there would be no significant difference in the performance of these animals on the pre-treatment probes. As predicted, there was no main effect of treatment and no quadrant x treatment effects ($F(2, 21)=2.207$, $P=0.14$, $F(2, 21)=2.158$, $P=0.14$) (Fig.5).

There were significant differences between the time spent in the cued quadrant and the average of time spent in the three un-cued quadrants for the probe ($F(1, 21)=35.687$, $P>0.001$). In this probe, only the hotplate was turned 180° while the cues and the aviary geometry remained consistent with training, therefore, the NW quadrant (the cued quadrant) was indicated by the cues, door and aviary geometry while the SE quadrant was indicated by the hotplate cues. There was a strong bias for the NW quadrant over all other quadrants combined. This indicates the birds were using the cues or aviary geometry and not features of the hotplate to learn the escape location. These results suggest all the birds learned the task equally.

The results indicate that propranolol administered acutely after reactivation does not affect the birds' memory on this task on recall trials. Since we failed to see an effect of propranolol in overall maze running ability, we wondered if there was no effect because propranolol did not affect spatial memory or because it did not affect some other type of memory the birds were relying on to complete the task. We wanted to make sure we were testing spatial memory specifically, so in subsequent experiments we examined pre-treatment probe performance versus post-treatment probe performance rather than the time to escape on recall trials. The recall trial results told us that performance was the same after propranolol treatment, but not whether differences were specific to spatial memory per se, as other cues could have been used during recall trials to complete the task.

EXPERIMENT 2

The results from Experiment 1 indicate that 20 mg/kg and 40 mg/kg of propranolol had no effect on spatial memory reconsolidation while 80 mg/Kg had side effects and still did not appear to alter spatial memory. Therefore, we decided to increase the dosage of propranolol to 60 mg/kg. Given that the injections 5 minutes after reactivation in experiment 1 did not appear to interfere with memory, we also wanted to explore whether the timing of injections might influence memory reconsolidation. Therefore, we decided to inject the birds 0 minutes post reactivation and 25 minutes post reactivation as experiments in rats showed injections 0 minutes post reactivation inhibited spatial memory reconsolidation (Cahill et al., 2000) and experiments in chicks showed injections 25 minutes post reactivation inhibited passive avoidance memory (Gibbs et al., 2005). In Experiment 1, we might have over-trained the birds on the spatial task making it more difficult to interfere with reconsolidation, so we decreased the amount of training trials from 16 to 10. The probes conducted in this experiment also differed from the probes in Experiment 1. In Experiment 1, the hotplate was rotated 180° to test if the birds were using the proximal cues of the hotplate to find the escape location. In Experiment 2, we changed the methods of conducting the probe trials in order to determine if the birds were using one of several types of cues, such as the hotplate, distal cues or room geometry, or a combination of the cues to locate the escape hole.

Method

Subjects

Just prior to the experiment, 18 naïve female zebra finches similar in age were selected at random from a single-sex aviary, weighed, and housed in individual carrying cages for easier access to birds during experimentation.

Behavioral Testing

The birds were divided into 2 groups of 9 based on the timing of drug injections. All 18 birds underwent spatial training in the Day Escape Maze as in Experiment 1, except each bird was given a total of 6 training trials on the first day, 1 probe trial on the second day, followed by 4 more training trials for each bird to ensure spatial learning had occurred. Two more probe trials were conducted on the third day, then a reactivation trial on the fourth day, then a final set of probe trials on the fifth day.

Probe Trials

On the second day of experimentation, a probe trial was conducted. Probe 1 involved replacing the escape cylinder with the no-escape cylinder and rotating the distal cues on the aviary walls 180°. This probe trial had the effect of putting several types of cues at odds with one another. If the birds were using the distal cues to learn the escape location during training, they would search for the escape hole in the southeast quadrant on the probe trial. If they were using aviary geometry to learn the escape hole location during training (Cheng, 1986), they would search in the northwest

quadrant during the probe trial. If they were using local cues to identify the escape hole during training, they would also search in the northwest quadrant. If the birds had been using multiple cue types, they might show mixed biases depending on the bird and its particular strategy. During Probe 1, the birds were placed in the maze at the south point and remained in the maze for 2 minutes. EthovisionTM was used to record the distance, latency and velocity in each quadrant. On the third day following 4 more training trials, 2 more probe trials were conducted. Probe 2 was the same as Probe 1. Probe 3 involved replacing the escape cylinder with a no-escape cylinder and rotating the entire aviary 180°, resulting in dissociation of local cues on the hot plate with room geometry and distal cues which were rotated together.

Reactivation, Injections, and Final Probes

On the fourth day, the birds were randomly assigned to 1 of 4 groups: 3 birds for saline 0.9% injections at 0 or 25 minutes post reactivation trials and 6 birds for propranolol 60 mg/kg injections at 0 or 25 minutes post reactivation trials. As in experiment 1, birds were run in a morning and afternoon batch. Following injections, all birds were returned to their isolation cages. On the fifth day, all 18 birds underwent 2 more probe trials. Probe 4 was conducted by replacing the escape cylinder with the no-escape cylinder, and Probe 5 involved replacing the escape cylinder with the no-escape cylinder and rotating the entire aviary 180° as in Probe 3. Data was recorded with Ethovision, and the same measures were collected as for the training trials.

Data Analysis

For this experiment, we had only 3 saline subjects in the 0 minute after injection and the 25 minute after injection groups. We did not expect any differences in performances between these 2 control groups. Thus, the first statistics we did were simply to validate combining these 2 groups. To do so, we used a 3-way repeated measures general linear model (GLM; treatment x injection time x trials) to examine differences in distance, latency, and velocity to escape. When there were no interactions of injection time with treatment, we combined the two saline groups. We then performed a 2-way repeated measures GLM (treatment x trials) to test for any differences in distance, latency and velocity between birds that would later be assigned to treatment groups on performance during the pre-treatment training. Measures of velocity are used to determine if there are differences in motor abilities among birds. For within subject variables, we examined Levene's test for equality of variance. To determine if there was a spatial bias and, if so, for which quadrant, for each probe trial, we confirmed using 3-way repeated measures GLM that the injection time x treatment control birds could be combined. Then, we used a 2-way repeated measures GLM, to test for differences in quadrant searching and between treatment groups in preference for quadrants. For probe trials, we used distance data to calculate quadrant preference values. Using distance in each quadrant rather than time has the advantage of not being influenced by any differences in velocity. For the distance data, we calculated the

proportion of total distance travelled in each quadrant (proportion = distance in quadrant/total distance in all quadrants). In order to improve the fit to normality, the proportions were transformed using the linear transformation (arcsin-squareroot). If there was a main effect of quadrants, differences in the quadrants searched were examined using planned comparisons among quadrants. Any treatment x quadrant interactions would be followed by a one-way repeated measures ANOVA for each treatment to examine spatial biases for that group. We did a planned comparison between the last trial of training and the reactivation trial to confirm that latency and distance to escape on the reactivation trial was similar if not better than the last trial of training, demonstrating memory was similar to training during reconsolidation. All P values shown for multiple t-tests were corrected using sequential Bonferroni correction. Finally, to determine the effect of the drug on spatial memory, the proportion of distance covered in each quadrant for identical types of probe trials that were run before and after drug treatment were compared using a nested two-way repeated measures GLM (quadrants nested in probes before and after treatment x treatment). Treatment effects on spatial memory would be indicated by interactions between treatment, probes, and quadrants.

Results and Discussion

Training

Analyses with 0 and 25 saline groups

Tests of sphericity were significant for distance ($\chi^2(54)=178$, $P<0.0001$), latency ($\chi^2(54)=87$, $P=0.006$), and velocity ($\chi^2(54)=101$, $P<0.0001$). Thus, we used the Greenhouse-Geisser correction factor to determine significance for within-subject effects. There was no main effect of injection time, and no interaction between treatment and injection time, treatment and trials, or the three way interaction between injection time, treatment, and trials for any of the three variables (distance: injection time $F(1,14)=0.566$, $p=0.464$, treatment x injection time $F(1,14)=0.642$, $p=0.436$, treatment x trials $F(2.984,41.783)=0.715$, $p=0.55$, 3-way interaction $F(2.984,41.783)=0.466$, $P=0.71$; latency: injection time $F(1,14)=0.059$, $p=0.81$, treatment x injection time $F(1,14)=0.250$, $p=0.63$, treatment x trials $F(4.162,58.273)=0.2$, $p=0.942$, 3-way interaction $F(4.162,58.273)=0.99$, $p=0.42$; velocity: injection time $F(1,14)=0.498$, $p=0.49$, treatment x injection time $F(1,14)=0.022$, $p=0.88$, treatment x trials $F(3.897,54.559)=0.993$, $p=0.42$, 3-way interaction $F(3.897,54.559)=0.942$, $p=0.45$). Since there was no effect of injection time for the saline animals, we combined these 2 groups for further analysis.

Combined Saline Group Analysis

Tests of sphericity were significant for distance ($\chi^2(54)=217$, $P<0.0001$) and latency ($\chi^2(54)=122$, $p<0.0001$), but insignificant for velocity ($\chi^2(54)=77$, $p=0.38$). Thus, we used the Greenhouse-Geisser correction factor to determine the significance for within-subject effects for distance and latency. During training, there had not yet been

any manipulation of the subjects, and birds had been randomly assigned to treatment groups. Thus for training trials we predicted there would be no significance difference in the learning between the animals that would be assigned to drug groups prior to treatment. There was no significant difference for the main effect of treatment nor any trial x treatment effects. Thus, random selection of birds was effective in equating performance across treatments during training for distance [Treatment: $F(2,15)=0.052$, $p=0.95$; Treatment by trial: $F(6.098,45.732)=0.477$, $p=0.83$], latency [Treatment: $F(2,15)=0.369$, $p=0.70$; Treatment by trial: $F(8.319,62.395)=0.353$, $p=0.95$], and velocity [Treatment: $F(2,15)=0.503$, $p=0.62$; Treatment by trial: $F(7.940,59.548)=0.675$, $p=0.71$].

It was also important that we confirm all groups learned the task prior to drug treatment. Levene's test was significant at or below $p=0.05$ for 3 trials out of 11 for distance, and 1 out of 11 trials for latency and velocity. This was indeed the case, as evidenced by a significant main effect of trials for distance ($F(3.049, 45.732)=23.256$, $p<0.0001$) and latency ($F(4.160, 62.395)=33.585$, $p<0.0001$) to escape. Furthermore, this main effect was due to a linear decrease in distance and latency as seen in the linear contrast effect ($F(1,15)=56.212$, $p<0.0001$; $F(1,15)=168.462$, $p<0.0001$, respectively) and in Fig. 6. Velocity differed across trials ($F(3.970,59.548)=3.106$, $p=0.02$). However the linear contrast of velocity across trials was not significant, (Fig. 6) ($F(1,15)=1.760$, $P=0.204$), suggesting this variation in velocity across trials, while significant, was unrelated to learning.

Pre-Treatment Probes and Reactivation

Initial Analysis

For this experiment, the birds were given a single probe trial following 6 training trials, 2 consecutive probe trials following 4 more training trials, and 2 more consecutive probe trials 24 hours post reactivation. For probes 1, 2, and 4 we rotated the distal cues on the aviary wall to test whether the birds were using these distal cues to find the location of the escape hole. Probes 3 and 5 involved rotating the entire aviary to determine if the birds were using the geometric shape of the room to find the location of the escape hole. These cue configuration disruption type of probe trials did not significantly affirm that the birds had learned the spatial maze. The birds seemed to vary in preference for using the distal cues, room geometry, and local cues on the hotplate.

We found that on all pre-treatment probes, the tests of sphericity were significant for quadrant (Probe 1: $\chi^2(5)=17$, $P=0.005$,; Probe 2: $\chi^2(5)=17$, $P=0.005$; Probe 3: $\chi^2(5)=21$, $P<0.0001$), and therefore, we used the Greenhouse-Geisser correction factor to determine significance for within-subject effects. There was no main effect of injection time, and no interaction between treatment and injection time, treatment and quadrant, or the three way interaction between injection time, treatment, and quadrant for distance on any of the pre-treatment probes (Probe 1: $F(1, 12)=0.091$, $p=0.77$, $F(1, 12)=0.108$, $p=0.75$, $F(1.522, 18.269)=1.411$, $p=0.26$, $F(1.522, 18.269)=2.698$, $p=0.11$; Probe 2: $F(1, 12)=0.031$, $p=0.86$, $F(1, 12)=0.120$, $p=0.74$, $F(1.755, 21.059)=0.413$,

p=0.641, $F(1.755, 21.059)=3.37$, $p=0.06$; Probe 3: $F(1, 12)=0.069$, $p=0.80$, $F(1, 12)=0.256$, $p=0.62$, $F(1.778, 21.335)=0.249$, $p=0.76$, $F(1.778, 21.335)=0.949$, $p=0.39$). Since injection time and not injection time interactions yielded significant effects, saline animals from the 2 groups were combined.

Combined Saline Group Analysis

Tests of sphericity were significant for ANOVAS on all probes (Probe 1: $\chi^2(5)=19$, $P=0.002$; Probe 2: $\chi^2(5)=23$, $P<0.0001$; Probe 3: $\chi^2(5)=22$, $P=0.001$). Thus, we used the Greenhouse-Geisser correction factor to determine the significance for within-subject effects for the dependent measures. Since these probe trials occurred before any manipulation of the subjects, we predicted there would be no significant difference in the performance of these animals on the pre-treatment probes. As predicted, there was no main effect of treatment and no quadrant x treatment effects. (Probe 1: treatment $F(2, 13)=0.471$, $p=0.63$, quadrant x treatment $F(3.001, 24.876)=1.629$, $p=0.22$; Probe 2: treatment $F(2, 13)=0.032$, $p=0.97$, quadrant x treatment $F(3.159, 20.524)=0.414$, $p=0.75$; Probe 3: treatment $F(2, 13)=1.247$, $p=0.32$, quadrant x treatment $F(3.676, 23.894)=1.318$, $p=0.29$). Thus, there was no significant difference in search patterns of birds prior to drug treatment.

It was important to assess spatial memory for the escape location prior to drug treatment so that we could determine any alterations in the effects of drug treatment on spatial memory. There were significant differences among the quadrants for Probe 1

($F(1.500, 24.876)=7.426, p=0.01$) and Probe 2 ($F(1.579, 20.524)=6.746, p=0.01$) but not for Probe 3 ($F(1.838, 23.894)=0.479, p=0.61$) (Fig. 7). Post hoc tests revealed that for Probe 1, the NW quadrant differed significantly from the SE and SW quadrants ($p=0.004$ and $p=0.01$, respectively) but not the NE quadrant ($p=0.18$). The NE quadrant also significantly differed from the SE quadrant ($p>0.001$). This was a cue configuration alteration trial so the SE quadrant was indicated by the rotated distal cues and the NW quadrant was indicated by aviary geometry and by the local cues on the hotplate. The birds' bias was for the NW quadrant, but there was some bias for the NE as well with very little searching in the SE or SW quadrants. This indicates birds were most likely using the door and room geometry over the distal cues. The NE search may indicate some disruption in spatial understanding for birds that had been using configuration of cue types during training, resulting in an altered trajectory. Probe 2 was conducted the same as Probe 1 but after 4 more training trials. Again, the NW differed significantly from the SE and SW ($p=0.001$ and $p>0.001$, respectively) but not the NE quadrant ($p=0.49$). The NE quadrant also significantly differed from the SE quadrant ($p=0.05$). Again, the general bias was for the NW quadrant but several birds spent more time in the NE than the NW indicating disrupted configural learning. On Probe 3, the aviary was turned while the hotplate and room geometry remained consistent with training. There was no main effect of quadrant on this probe indicating the animals searched each quadrant similarly. This may have occurred because this probe trial directly followed

Probe 2, during which some animals appeared to have used configural learning of cues and some birds that appeared to have been attending to the room geometry which is was now back in line with the distal cues but out of synch with the local cues on the hotplate, possibly altering these animals' ability to define the spatial location in this configural distortion. Probe 1 suggests most of the birds were able to learn the spatial location of the escape hole after only 6 training trials in one day, but tend to use room geometry cues over distal cues. Probe 2 confirmed that after 4 more training trials, the birds still consistently used their preference of spatial cues to identify the escape hole. Probe 3 results could suggest that spatial learning was not strong after Probe 2, possibly because of extinction effects occurring during Probe 2, when there was a lack of escape opportunity. However, it is more likely that this Probe 3 shows us that different animals are using different cues, and that the dissociation of these cues leads to an overall group appearances of non-preference for any one quadrant. Because animals did not unanimously follow a particular cue type, it was necessary for us to examine an individual's performance before and after drug treatment on identical probe types. In doing so, we should be able to interpret any changes in performance as drug effects.

Reactivation

Planned comparisons revealed no differences between the last training trial (Trial 10) and the Reactivation Trial (Trial 11) for distance ($P=0.38$), latency ($P=0.21$) or velocity ($P=0.21$). This suggests there was no decrease in task competence between

these trials and implies that memory was indeed reactivated on the Reactivation Trial. This also supports the conclusion that the probe trial performance was indentifying particular quadrant biases rather than any lack of spatial learning as realignment of all cue types on the activation trial demonstrated continued memory for the escape location.

Post-Treatment Probes

Tests of sphericity were significant for quadrant effects on Probe 5 ($\chi^2(5)=18.477$, $p=0.002$) but not Probe 4 ($\chi^2(5)=9.943$, $P=0.08$). Greenhouse-Geisser correction factor was used to determine the significance for within-subject effects for the dependent measures for Probe 5 only. There was no main effect of treatment and no quadrant x treatment effects on either probe (Probe 4: $F(2, 13)=0.996$, $p=0.40$, $F(4.335,28.306)=0.519$, $p=0.74$; Probe 5: $F(2, 13)=2.101$, $p=0.16$; $F(3.042,19.773)=0.664$, $p=0.59$).

Probe 4 involved replacing the escape cylinder with a no-escape cylinder, and Probe 5 involved replacing the escape cylinder with a no-escape cylinder and rotating the entire aviary 180°. There were significant differences between the quadrants for Probe 4 ($F(2.177,28.306)=6.615$, $p=0.004$) but not on Probe 5 ($F(1.521,19.773)=2.249$, $p=0.141$) (Fig. 8). Post hoc tests using the Bonferroni correction revealed that for Probe 4, the NW quadrant differed significantly from the NE, SW, and SE quadrants ($p=0.004$, $p=0.03$, and $p=.006$, respectively). This indicates that post-treatment all the birds

searched for the escape in the quadrant that indicated the escape hole via distal cues. For Probe 5, there was no effect of quadrant indicating the animals searched each quadrant equally. While there was not a treatment effect for Probe 5, post hoc tests for quadrant effects show there was a general bias for the NW quadrant with most animals searching that quadrant, but there was still some bias for the SE quadrant indicating these animals were using the hotplate features and not the cues.

Drug Treatment Effects

We compared quadrant searching behavior of drug treatment groups in identical probe types before and after drug treatment (Probe 3 versus Probe 5 – aviary was rotated with distal cues indicating escape hole in the SE quadrant, while room geometry and local cues on the hotplate were consistent with the escape hole in NW quadrant). There was no main effect of treatment, and no probe x treatment effects (Treatment: $F(2,13)=2.230$, $P=0.15$; Probe x Treatment: $F(2,13)=0.665$, $P=0.53$) (Fig. 9), indicating that the drug had no effect on memory of the task. Although there were no significant interactions, there were some differences in the groups. While the saline birds retained the same bias pre and post-treatment, the Prop 0 groups shifted from a strong SE to strong NW bias, and the Prop 25 groups shifted from a strong NW bias to a divided bias between NW and SE. This may indicate a shift in which maze features (i.e. cues versus hotplate) the birds were utilizing in the drug treatment groups but not in a very

predictable manner, with Prop0 improving spatial bias while Prop25 decreasing spatial bias.

EXPERIMENT 3

Experiment 1 and Experiment 2 tested the effect of β -AR antagonists on spatial memory reconsolidation and showed no effect. Therefore, we used an α -AR antagonist to test if α -ARs play a role in spatial memory reconsolidation. It is known that Phentolamine, an α -AR antagonist, inhibits song-induced gene expression and significantly decreases song ability in zebra finches (Velho et al., 2012). In addition, α -adrenergic receptors are known to be expressed in the song system of zebra finches (Riters et al., 2002) and appear to be present in the hippocampus (personal communication Lauren Riters November, 2013). If the actions of ARs are conserved in spatial memory reconsolidation in rodents and zebra finches, phentolamine should impair memory consolidation when administered near reactivation of a spatial memory. To test this, we injected Phentolamine at 0 minutes post reactivation or 25 minutes post reactivation after spatial training as described above for Experiment 2.

Method

Subjects

As in Experiment 2, 18 naïve female zebra finches were selected as random from a single-sex aviary, weighed, and housed in individual carrying cages just prior to experimentation.

Behavioral Testing

As in Experiment 2, the 18 birds were divided into 2 groups of 9 based on the timing of drug injections. All 18 birds underwent spatial training in the Day Escape Maze as in Experiment 1, except for differences in the probe trials. In this experiment, each bird was given a total of 6 trials on the first day, 2 probe trials were conducted for each bird on the second day, then 4 more training trials for each bird were conducted on the second day to ensure spatial learning had occurred. Probes are described below.

Probe Trials

Probes were conducted similarly to Experiment 2 with the following exceptions. The first and second probe trials were conducted on day 2. Probe 1 involved replacing the escape cylinder with a cylinder lacking the escape hole. Probe 2 was conducted by replacing the escape cylinder with the no escape cylinder and rotating the stool and hotplate 180°, so that local cues would indicate escape in the quadrant 180° from that indicated by the distal cues and room geometry. During both probes, the birds were placed in the maze at the south point and remained in the escape maze for 2 minutes. We used Ethovision software to record the distance in each quadrant. On the third day after 4 more training trials, Probe 3 and Probe 4 were conducted in the same manner as the Probe 1 and Probe 2 trials before.

Reactivation, Injections, and Final Probes

On the third day of experimentation following the probe trials, the birds were randomly assigned to 1 of 4 groups: saline 0.9% injections at 0 (n=3) or 25 (n=3) minutes

post reactivation trials and Phentolamine 40 mg/kg at 0 (n=6, Phen 0) or 25 (n=6, Phen 25) minutes post reactivation trials. As in Experiment 1, birds were run in a morning and afternoon batch. All 18 birds were returned to their isolation cages following injections. On the fourth day, Probe 5 and Probe 6 were conducted in a manner consistent with Probes 1 and 2 and Probes 3 and 4 for all 18 birds. Data was again recorded with Ethovision.

Data Analysis

Data analysis for this experiment is the same as in Experiment 2, except that the probes that were identical differed between the two experiments. Final comparisons of probe trials to determine drug treatment effects involved comparisons of Probes 3 and 5 and Probes 4 and 6.

Results and Discussion

Training

Analyses with 0 and 25 saline groups

The Mauchly's test of sphericity was significant for distance ($\chi^2(54)=194$, $p<0.0001$) and latency ($\chi^2(54)=146$, $p<0.0001$). Thus, we used the Greenhouse-Geisser correction factor to determine significance for within-subject effects for these dependent measures. The test of sphericity was not significant for velocity ($\chi^2(54)=74$, $p=0.058$). There was no main effect of injection time, and no interaction between treatment and injection time, treatment and trials, or the three way interaction

between injection time, treatment, and trials for any of the three variables (distance: $F(1,14)=0.159$, $p=0.70$, $F(1,14)=0.078$, $p=0.78$, $F(3.665, 51.312)=2.363$, $p=0.70$, $F(3.665, 51.312)=0.505$, $p=0.72$; latency: $F(1,14)=3.251$, $p=0.09$, $F(1,14)=2.174$, $p=0.16$, $F(4.034, 56.476)=1.398$, $p=0.25$, $F(4.034, 56.476)=0.725$, $p=0.58$; velocity: $F(1,14)=1.948$, $p=0.19$, $F(1,14)=0.563$, $p=0.47$, $F(4.479, 62.704)=1.275$, $p=0.29$, $F(4.479, 62.704)=0.824$, $p=0.53$). Since there was no effect of injection time x treatment, we combined the saline animals into one group.

Combined Saline Group Analysis

The test of sphericity was significant for distance ($\chi^2(54)=217$, $p<0.0001$), latency ($\chi^2(54)=122$, $p<0.0001$), and velocity ($\chi^2(54)=77$, $p=0.04$), so the Greenhouse-Geisser correction factor was used. Levene's test was significant at or below $p=0.05$ for 6 trials out of 11 for distance, 8 out of 11 for latency, and 7 out of 11 for velocity. During training, there had not yet been any manipulation of the subjects, and birds had been randomly assigned to treatment groups. Thus for training trials, we predicted there would be no significant difference in the learning of these animals pre-treatment. There was no significant main effect of treatment for distance $F(2, 15)=0.602$, $p=0.56$, or velocity $F(2,15)=3.029$, $p=0.08$. However, there was a significant difference in latency $F(2,15)=5.207$, $p=0.02$ with the Phen 25 group differing significantly from the saline and Phen 0 groups (post-hoc tests, $p=0.02$ and $p=0.01$, respectively). The marginally significant difference in velocity may have contributed to this difference in latency

rather than differences in learning. Regardless, this difference was not due to treatment, as the animals had not yet been treated. Instead, it shows that random assignment of birds to groups did not result in equal potential for learning. Because each birds' performance will be compared to itself when analyzing drug treatment effects, this difference in groups should not distort the interpretation of the results.

All groups learned the task prior to treatment, as evidenced by a significant main effect of trials for distance ($F(3.572, 53.582)=23.186$, $p<0.0001$) and latency ($F(3.954, 59.313)=33.609$, $p<0.0001$) to escape. Velocity ($F(4.638, 69.575)=3.344$, $P=0.01$) differed across trials as well. The main effect of trials showed a significant linear contrast indicating a decrease in distance and latency across trials ($F(1, 15)=81.489$, $p<0.0001$; $F(1, 15)=105.46$, $p<0.0001$, respectively) and in Fig. 10. While velocity varied across trials ($F(4.638, 69.575)=3.344$, $p=0.01$), this variation was not linear ($F(1, 15)=0.027$, $p=0.87$) suggesting changes across trials were unrelated to learning.

Pre-Treatment Probes and Reactivation

Initial Analysis

We found that on all pre-treatment probes, the test of sphericity was significant for distance (Probe 1: $\chi^2(5)=28$, $p<0.0001$; Probe 2: $\chi^2(5)=10$, $p=0.076$; Probe 3: $\chi^2(5)=20$, $p<0.0001$), and therefore, we used the Greenhouse-Geisser correction factor to determine significance for within-subject effects. There was no main effect of injection time, and no interaction between treatment and injection time, treatment and

quadrant, or the three way interaction between injection time, treatment, and quadrant for the proportion of distance traveled in quadrants on any of the pre-treatment probes (Probe 1: $F(1, 14)=0.274$, $p=0.61$, $F(1, 14)=3.482$, $p=0.08$, $F(1.622, 22.713)=3.011$, $p=0.08$, $F(1.622, 22.713)=0.462$, $p=0.62$; Probe 2: $F(1, 14)=0.394$, $p=0.54$, $F(1, 14)=0.675$, $p=0.42$, $F(2.240, 31.360)=2.031$, $p=0.14$, $F(2.240, 31.360)=1.566$, $p=0.22$; Probe 3: $F(1, 14)=0.394$, $p=0.54$, $F(1, 14)=0.675$, $p=0.42$, $F(2.240, 31.360)=2.031$, $p=0.14$, $F(2.240, 31.360)=1.566$, $p=0.22$). Since injection time did not affect performance, saline animals from the 2 groups were combined.

Combined Saline Group Analysis

Tests of sphericity were significant for quadrant on Probe 1, 2, and 3 (Probe 1: $\chi^2(5)=30$, $p<0.0001$; Probe 2: $\chi^2(5)=11$, $p=0.05$; Probe 3: $\chi^2(5)=21$, $p<0.0001$) but not Probe 4 ($\chi^2(5)=7.983$, $p=0.16$). Thus, the Greenhouse-Geisser correction factor was used to determine the significance for within-subject effects for the dependent measures for probes where the test of sphericity was significant. Since these probe trials occurred before any manipulation of the subjects, we predicted there would be no significant difference in group performance. As predicted, there was no main effect of treatment and no quadrant x treatment effects. (Probe 1: $F(2, 15)=1.230$, $p=0.32$, $F(3.372, 25.293)=1.709$, $p=0.19$; Probe 2: $F(2, 15)=0.355$, $p=0.71$, $F(4.437, 33.278)=1.938$, $p=0.12$; Probe 3: $F(2, 15)=0.991$, $p=0.39$, $F(3.267, 24.506)=0.483$, $p=0.71$; Probe 4: $F(2, 15)=0.841$, $p=0.45$, $F(4.292, 32.190)=1.090$, $p=0.38$). Thus, despite some differences in

escape latency during training between the Phen 25 birds and the saline and Phen 0 birds, there were no group differences in spatial memory as indicated by a tendency to search particular quadrants of the maze during probe trials.

There were significant differences between the proportion of distance searched in the quadrants for all four probe trials (Probe 1: $F(1.686, 25.293)=5.987$, $p=0.01$; Probe 2: $F(2.219, 33.278)=7.834$, $p=0.001$; Probe 3: $F(1.634, 24.506)=6.818$, $p=0.01$; Probe 4: $F(2.146, 32.190)=4.586$, $p=.016$) (Fig. 11). Post hoc tests using the serial Bonferroni correction revealed that for Probe 1, the NW quadrant differed significantly from the SE and NE quadrants ($p=0.001$ and $p=0.002$, respectively) but not the SW quadrant ($p=0.13$). This split bias for both the NW and SW suggests the animals had not yet learned the location of the escape hole when the escape hole was removed and all other features remained consistent with training. For Probe 2, the hotplate was rotated 180° while all other features remained consistent. Again, the NW quadrant differed significantly from the SE and NE quadrants ($p=0.02$ and $p=0.0001$, respectively) but not the SW quadrant ($P=0.14$). This reinforces the suggestion that the animals did not yet have a strong spatial memory for the location of the escape hole. Probe 3 was conducted the same as Probe 1 but after 4 more training trials. For this probe, the NW quadrant differed significantly from the SE and NE quadrants ($p=0.0001$ and $p=0.0001$, respectively) but not the SW quadrant ($p=0.12$). Probe 4 was conducted the same as Probe 2 but after 4 more training trials. On this probe, the NW quadrant differed

significantly from the NE, SW, and SE quadrants ($p=0.01$, $p=0.01$, and $p=0.03$, respectively). This indicates that by the 4th probe trial all the birds demonstrated use of the distal cues to locate the escape quadrant when the hotplate was rotated and all other cues remained consistent. It is somewhat unclear why birds would perform in a more biased fashion on Probe 4 than on Probe 3 when, if anything, spatial information was more consistent with training in Probe 3 than in Probe 4 and when some extinction would accompany Probe 3 before performance in Probe 4. Nevertheless, this indicates that all birds had strong spatial memory prior to drug treatment.

Reactivation

A planned comparison revealed no differences between the last training trial (Trial 10) and the Reactivation Trial (Trial 11) for distance ($p=0.51$), latency ($p=0.10$) or velocity ($p=0.21$). This suggests there was no decrease in task competence between the last trial of training and reactivation and implies the memory was indeed reactivated on the Reactivation Trial.

Post-Treatment Probes

Tests of sphericity were significant for quadrant on Probe 5 ($\chi^2(5)=18$, $p=0.003$) and Probe 6 ($\chi^2(5)=26$, $p<0.0001$). Thus, Greenhouse-Geisser correction factor was used to determine the significance for within-subject effects for the dependent measures. There was no main effect of treatment and no quadrant x treatment effect on either

probe (Probe 5: $F(2, 15)=0.184$, $p=0.83$, $F(4.115, 30.863)=0.464$, $p=0.77$; Probe 6: $F(2, 15)=2.358$, $p=0.13$, $F(2.877, 21.580)=0.65$, $P=0.59$).

There were significant differences between the quadrants for Probe 6 ($F(1.439, 21.580)=5.164$, $p=0.02$) but not on Probe 5 ($F(2.058, 30.863)=1.279$, $p=0.29$) (Fig. 12). Post hoc tests using the serial Bonferroni correction revealed that for Probe 6, the NW quadrant differed significantly from the NE and SE quadrants ($p<0.0001$, and $p<0.0001$, respectively) but not the SW quadrant ($p=0.215$). This indicates that post-treatment, all the birds remembered the correct escape quadrant when the hotplate was rotated and all other cues remained the same suggesting the birds were using the cues and room features and not the hotplate features to locate the escape hole during training. While there was no significant differences between the treatment groups, the means suggested different patterns across trials for the groups. The saline group showed a mixed bias for NW and SW quadrants, the Phen 25 had the same split bias but it was more pronounced than the saline animals, and the Phen 0 group was most biased to the NW quadrant. For Probe 5, there was no effect of quadrant indicating the animals searched each quadrant similarly when the escape hole was removed and all other features of the aviary remained consistent with training. This is a bit unusual, as only one day before, prior to reactivation, the birds had a strong quadrant bias in an identical probe to Probe 5. These results do parallel those for the strength of bias in Probe 3 and Probe 4, which suggested that birds performed better when the hotplate is turned

rather than when all the cues are the same as they were during training and following what is basically an extinction trial in Probe 3 and Probe 5. How these processes would enhance rather than reduce spatial bias for the trained quadrant is unclear.

Drug Treatment Effects

We examined differences in quadrant performances before and after drug treatment between Probes 3 and 5 (escape hole removed, all other cues consistent probes), and Probes 4 and 6 (hotplate flipped, all other cues consistent probes). There was no significant main effect of treatment, and no probe x treatment effects for either Probe 3 vs 5 or Probe 4 vs 6 (Probe 3 vs 5: $F(2,15)=0.485$, $p=0.63$, $F(2,15)=0.483$, $p=0.63$; Probe 4 vs 6: $F(2,15)=1.611$, $p=0.23$, $F(2,15)=1.053$, $p=0.37$) (Fig. 13 and 14, respectively). This indicates the drug treatment had no effect on memory for the task.

GENERAL DISCUSSION

In our experiments, we aimed to determine whether NE plays a role in reconsolidation of spatial memory in zebra finches. Our results suggested that AR antagonists do not influence spatial memory reconsolidation in zebra finches, as evidenced by the consistent performance on recall trials in experiment 1 and consistent probe trial performances before and after administration of the β -AR antagonist propranolol in experiment 2. In addition, experiment 3 showed that phentolamine, an α -AR antagonist, also failed to affect spatial memory reconsolidation, as evidenced by a lack of treatment effect on probe trials conducted before and after drug treatment. Given the conservation of hippocampal function in spatial memory across vertebrates (Mayer et al., 2012), similarities in NE projections (Gibbs et al., 2010), AR distributions (Fernandez-Lopez et al., 1997), and similarities in mechanisms underlying memory processing (Tronson et al., 2007), it is surprising that our experiments did not support conservation of the role of NE on spatial memory reconsolidation in zebra finch.

We know that the hippocampus is involved in spatial learning in the zebra finch (Mayer et al., 2012), but we also know that spatial navigation involves multiple brain regions, which may facilitate in recall of the spatial memory if hippocampal ARs have been inhibited. However, the role of other brain regions in spatial memory is usually ancillary, such as the role of the cerebellum (Leggio et al., 1999) in learning procedures and the role of the parahippocampal areas in learning about the cues related to the goal

location (Breedlove et al., 2007). However, except under conditions of specialized training, animals that have hippocampal interference do not demonstrate use of distal cues to locate the goal. In our experiments, the probe trials showed that birds were using distal cues, which were the shapes on the aviary walls, to locate the escape location during training. It is still possible, however, that a role in reconsolidation of spatial memories is not as conserved across taxa as the role of the hippocampus in spatial learning.

Since hippocampal involvement in spatial navigation is conserved in zebra finches, perhaps it is the distribution of α - or β -ARs in the hippocampus of zebra finches that is not conserved. From previous studies, we know that propranolol and phentolamine act on ARs in the hippocampus of both rats (Cahill et al., 2000) and chicks (Gibbs et al., 2008), and we know that there are α -ARs in the zebra finch hippocampus (Riters et al., 2002). However, we also know that there is a great deal of variation in the distribution of ARs across avian groups. For example, a study comparing the AR type and distribution in the pigeon and chick revealed the densities of β -ARs throughout the brain varied between the species, with the exception of the cerebellum (Fernandez-Lopez et al., 1997). Also, no one has shown an effect of ARs in the hippocampus of spatial memory in birds, as the chick study examined passive avoidance learning (Gibbs et al., 2005). Thus, either there are no β -ARs in the zebra finch hippocampus and these are the ARs important for consolidation of spatial memory, or the role of the hippocampus in

the reconsolidation of spatial memory involves different neuronal mechanisms than in rats. To answer the first part of this question, we hope to follow these experiments by examining the distribution of α - or β -ARs in the zebra finch hippocampus. Then, we must consider that these ARs may not play a similar role in memory consolidation as they do in rats. We may need to examine a simpler task to elicit the role of ARs in hippocampally dependent behavior in birds. In chicks, for example, the hippocampus is involved in a color discrimination task (Gibbs et al., 2008). Perhaps this task would show conservation of ARs in consolidation of memory. Alternatively, we may have the right task but not the right timing window for interference with reconsolidation.

The process of reconsolidation is highly dependent on a precise interplay between receptors involved in LTP and receptors like ARs that modulate these processes. The timing of injections that are associated with subsequent memory inhibition reflects the time at which the ARs are required during memory processing (Gibbs et al., 2005). Injection timing has a direction correlation with STM, ITM, and LTM processes. Therefore, depending on the phase in which the spatial memory of the task was being processed at the time of injection, the injection of the AR antagonist may or may not have been able to interfere with memory consolidation. We know that in chicks performing a passive avoidance task, β 1-AR antagonists inhibit STM formation in the MSt when injected 10 minutes before training, β 3-AR antagonists inhibit ITM in the IMM when injected 5 minutes after training, and β 2-AR antagonists inhibit consolidation of

ITM into LTM in the IMM when injected 5 and 25 minutes post training (Gibbs et al., 2005). In rats, memory reconsolidation of the Morris Water Maze is inhibited when propranolol is injected immediately after the last training trial (Cahill et al., 2000). Our results and the evidence from these previous studies suggest that accurate timing of injections is complicated and depends on the brain region involved, species tested, and task demands. A study on the temporal processing of spatial memory consolidation in zebra finches has not yet been done, and therefore the optimal injection time to cause procedural amnesia of the spatial task is not known. We do know that LTP is important to zebra finch learning, as NMDA antagonists interfere with song learning (Basham et al., 1996), but further studies examining in detail the temporal elements of LTP in the zebra finch hippocampus would need to be done followed by experiments of NE antagonists and agonists layered on top of this process to determine how NE modulates LTP. Because the spatial memory task is quite complicated, interfering with reconsolidation of a single memory may not be easy.

In rats, where NE antagonists are known to interfere with consolidation of spatial memory in some experiments (Cahill et al., 2000, Gibbs et al., 2005), other studies suggest that these antagonists do not have an effect on spatial learning when given acutely to another rodent, such as mice (Czech et al., 2000). Instead, NE antagonists interfered with a spatial task only when given chronically and thus interfering with initial consolidation rather than reconsolidation. Similarly, propranolol effects on extinction of

reward running behavior, which measures the velocity in which an animal reaches a reward at the end of a straight runway after learning the reinforcement is there, in rats are only effective when injections are given chronically and not when given acutely (Terry et al., 1990). The results of these studies show that the effective injection timing of AR antagonists appears to vary with species and task. Therefore, we have run experiments similar to those reported here but with chronic daily treatment with α - and β -AR antagonists. Results remain to be analyzed.

While the hippocampus is involved in spatial memory, it is also involved in conscious memories for events and facts known as declarative memories (LaBar et al., 2006), such as configural and contextual memories. One possibility for unaltered spatial performance after drug treatment in our experiments is that the memory of the spatial task is no longer just spatial, but may also be influenced by contextual memory, which is created by learning the association between a novel environment and an aversive stimulus (Bach et al., 1995). In our experiments, a contextual memory of the entire aviary may have been created in response to the heat stimulus from the hotplate and the increased level of stress felt by the birds. One study shows that when spatial memory was inhibited in mice, they still performed normally in a contextual memory task, suggesting that even though both types of memory require the hippocampus, they may be mediated by different synaptic mechanisms (Bach et al., 1995). Therefore, antagonists that may affect the ARs involved in spatial memory may not affect other

types of memory in the hippocampus. So, the memory of the location of the escape hole may not be purely spatial, but may also involve other memories such as place recognition declarative memory. This possibility would suggest that the overall memory of the location of the escape hole involves multiple synaptic pathways and receptors, and therefore would require many different antagonists to prevent the actions of all the ARs on which hippocampal memories depend. Given that spatial memory is affected by NE antagonists in other species, if contextual memory is involved in zebra finch spatial learning, our results imply that either contextual learning or effects of NE on contextual memory are not conserved across species.

The possibility of evoking multiple types of hippocampal memories in finding the location of the escape hole suggests that reconsolidation of each one of these memories became stronger after each training trial. This would make a memory so strong that it would be hard to alter it during recall. However, our results show that the birds had not over-learned the spatial location of the escape hole in a strong fashion in all three experiments. The average escape time prior to treatment was about 20 seconds, whereas birds reach an asymptote of about 4 seconds if training proceeds for about 6 days and 4 trials a day (Pegues et al., 2013). In addition, some probe trials conducted prior to treatment did not show a significant bias for searching in a particular quadrant. If learning during training trials was weak or unstable, the behavior is less likely to be maintained during the probe trial (Czech et al., 2000), which suggests that some of our

probes were insignificant because of under-training. Thus, overtraining is not a likely explanation for a lack of treatment effect.

Our studies suggest that inhibition of memory by lowering NE influence on reconsolidation may not be a conserved mechanism across vertebrates. In chicks, emotional state does appear to affect memories as heightened emotional arousal due to maternal hen calls or predatory calls were suggested to increase memory retention (Field et al., 2007). Thus, it is surprising that we did not find an effect of treatment on reconsolidation given that the heat used to promote escape in the Day Escape Maze is likely to have some stress component. Our studies were propelled by studies with PTSD patients, which suggested that memories with emotional content are the hardest to forget, and that treatment with drugs that block the emotional enhancement of memories might contribute to forgetting.

In rats, humans, and chicks, heightened states of emotion facilitate and even amplify learning and memory (Roosendaal et al., 2011, Hu et al., 2007, Field et al, 2007). As noted previously, PTSD is an example how the stimulus initiating the memory process determines how strongly that memory is consolidated. The neuromechanism involved in PTSD involves the release of adrenal stress hormones in response to stressful or emotionally arousing events, which interact to facilitate memory formation and consolidation (Donovan, 2010). PTSD patients experience vivid memory recalls, which are enhanced after every reconsolidation, and which may be triggered by the smallest

onset. For example, a war veteran may become startled at the sound of a car door closing, because the noise recalls memories of war sounds. Based on studies on the cognitive neuroscience of PTSD (Donovan, 2010, LaBar et al., 2006), it is possible that the Day Escape Maze used in our experiments initiates this enhanced consolidation due to high stress levels and arousal experienced by the birds. The birds initially showed signs of panic when placed in the arena on a hotplate and were unaware of the location of the escape hole. Therefore, even when location of the escape hole was learned, the birds may experience the same stressed feeling when placed in the maze again, which would cause the reconsolidated spatial memory to become more enhanced in each trial. This enhanced reconsolidation, along with multiple stimuli in the Day Escape Maze, which could recall this memory, may make the antagonists used in our experiments less effective in inhibiting spatial memory. However, some zebra finches appear non-plussed by the maze and calmly walk around the maze for two minutes with no evidence that the heat stimulus is painful or stressful. Also, we expected our treatments to ameliorate any stressful elements of the maze and act on ARs in the hippocampus to decrease reconsolidation of any stressful components. We have posted various reasons why this might be the case, but at this time, it is unclear why this mechanisms might not be conserved.

In the future, we plan on conducting more experiments based on the current understanding of our results and possible explanations for why the AR antagonists did

not have an effect on spatial memory in zebra finches. Currently, we are finishing the analysis of an experiment in which birds were injected chronically with propranolol and phentolamine. Following this experiment, we plan on conducting experiments further investigating the effects of propranolol and phentolamine when injected acutely, in hopes to find the optimal injection time for the antagonists. The training and probes trials will be conducted in a more consistent manner and will prevent the possibility of extinction, as previously discussed. Also, we will investigate the presence and distribution of adrenergic receptors in the hippocampus of the zebra finch, which will help us determine if these receptors are distributed differently in mammals than in birds or, instead, operate differently in reconsolidation in avian brains than in mammalian brains.

Conclusion

A norepinephrine antagonist does not produce amnesic effects on spatial memory in a zebra finch given 0 minutes or 25 minutes after reactivation of the consolidated memory. The effects of norepinephrine on spatial memory or the distribution of adrenergic receptors may not be conserved in birds, but leaves open the possibility that norepinephrine is involved in other types of learning and memory in zebra finches.

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FIGURES

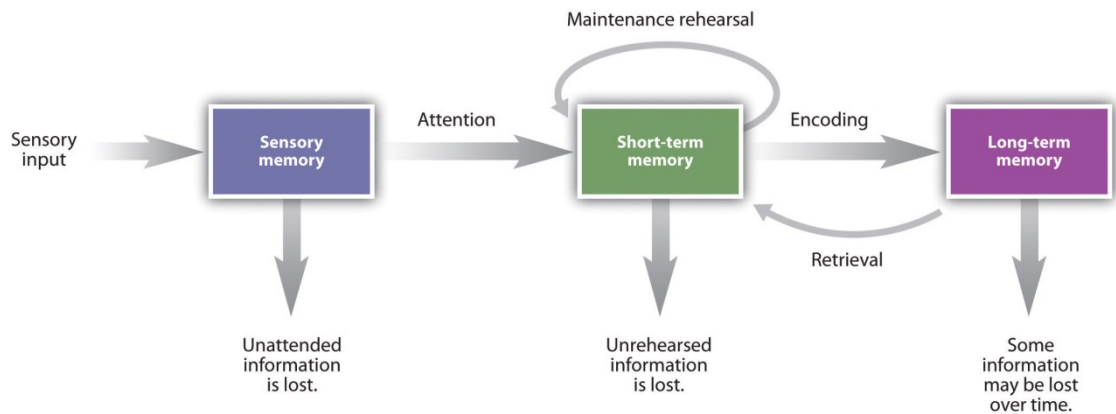


Figure 1. Stages of memory. Memory can be characterized by the length of time the information remains available for recall. Information begins as a sensory memory then through attention moves to short-term memory then to long-term memory. Sensory memory is extremely brief and without attention, will be forgotten in seconds. Short-term memory is where small amounts of information can be held for a little longer but only up to about one minute. For information to be available for recall after a minute, it must be encoded in long-term memory where it can be retrieve for days, months, and even years. (Adapted from: Atkinson & Shiffrin, 1968)

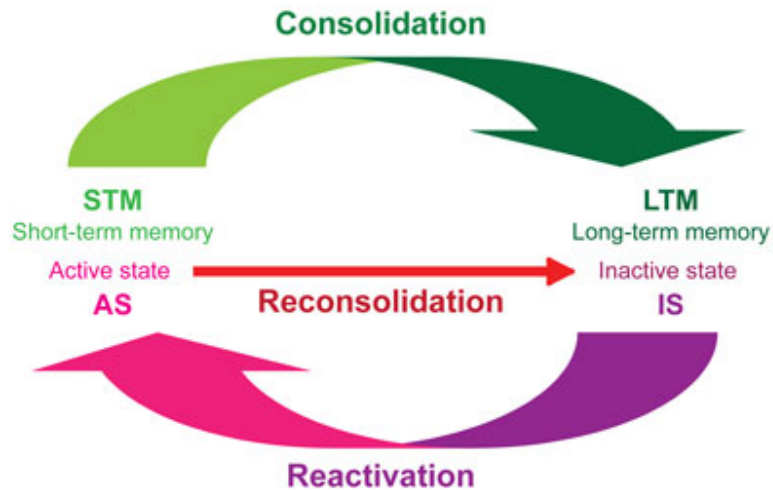


Figure 2. Reconsolidation of memory. The Reconsolidation Hypothesis states that following reactivation established memories become labile and then require another phase of protein synthesis in order to be maintained. Therefore, it has been proposed that each time a memory is reactivated it again undergoes a process of stabilization, named reconsolidation. (Adapted from: Inda, Muravieva, & Alberini, 2011)

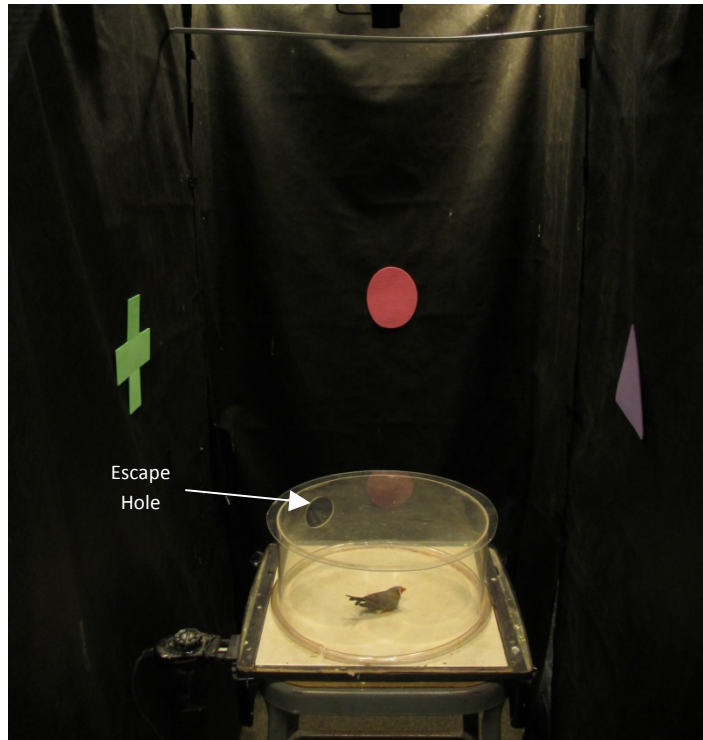


Figure 3. The Day Escape Maze. Pictured above is the Day Escape Maze, viewed from the entrance facing the Northern end of the aviary. Not pictured is the yellow star on the door of the aviary.

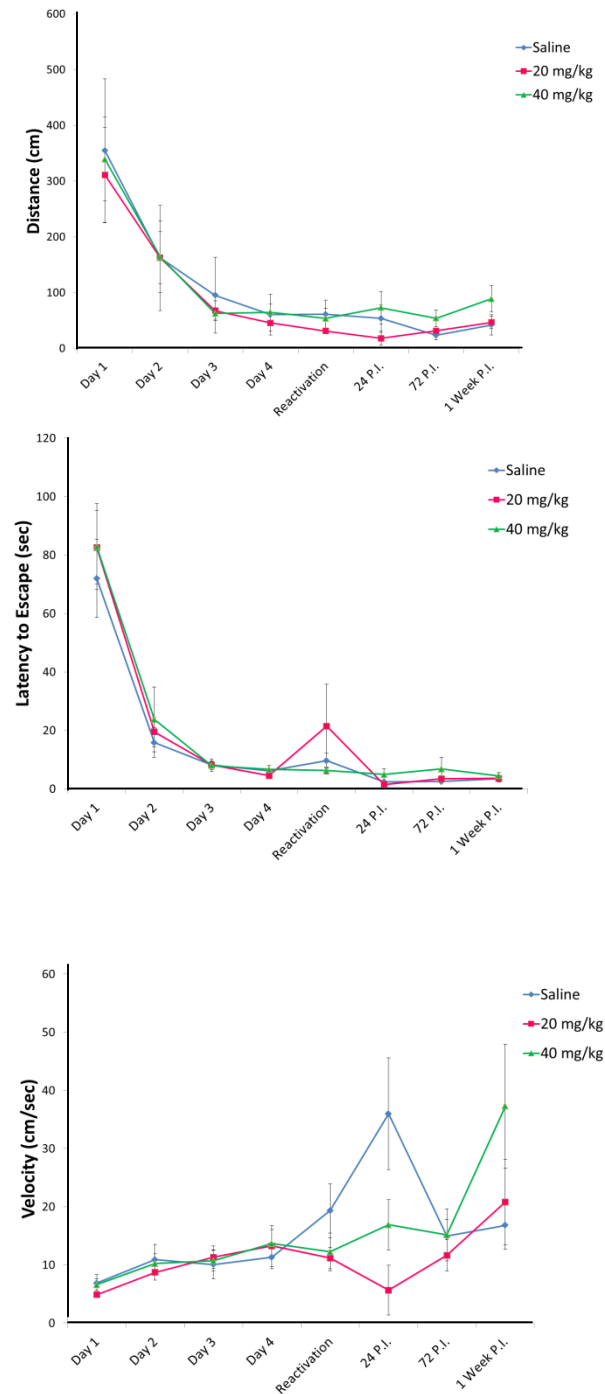


Figure 4. Average distance, latency of escape, and velocity by treatment condition over trial days. If trial day contained more than a single trial (Day 1-4), those trials were averaged to give a trial block or day average.

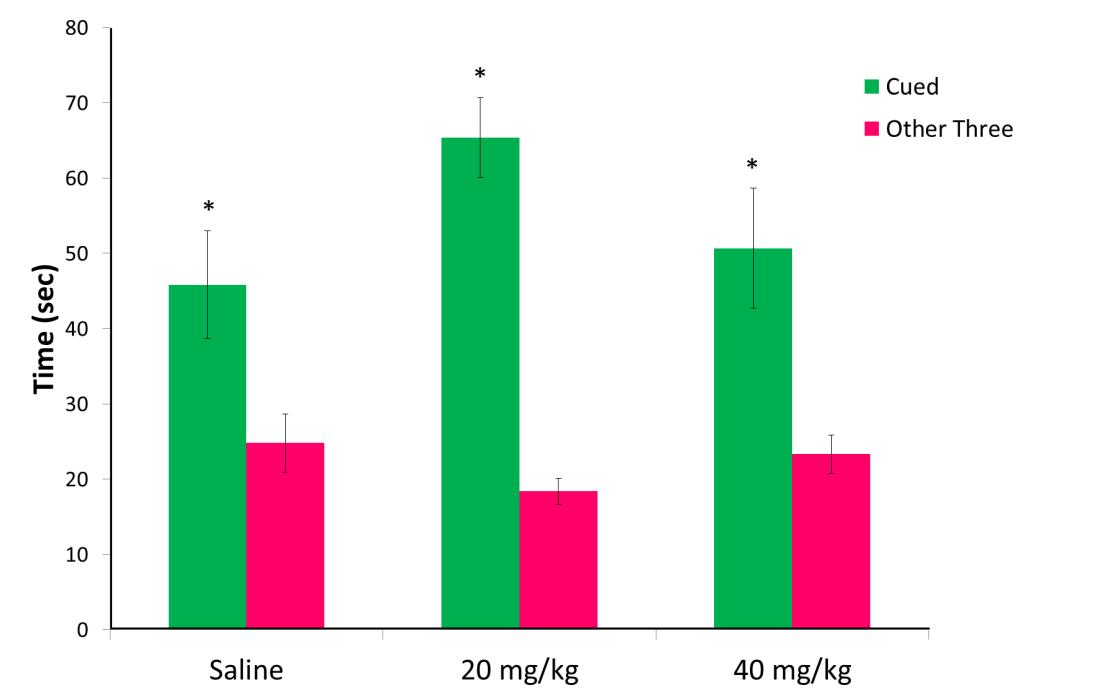


Fig. 5. Pre-treatment Probe. Comparison of time spent in the cued (correct) quadrant versus time spent in all non-cued quadrants. Asterisks indicate a significant difference between the mean time spent in the cued quadrant versus the non-cued quadrants ($P<0.05$).

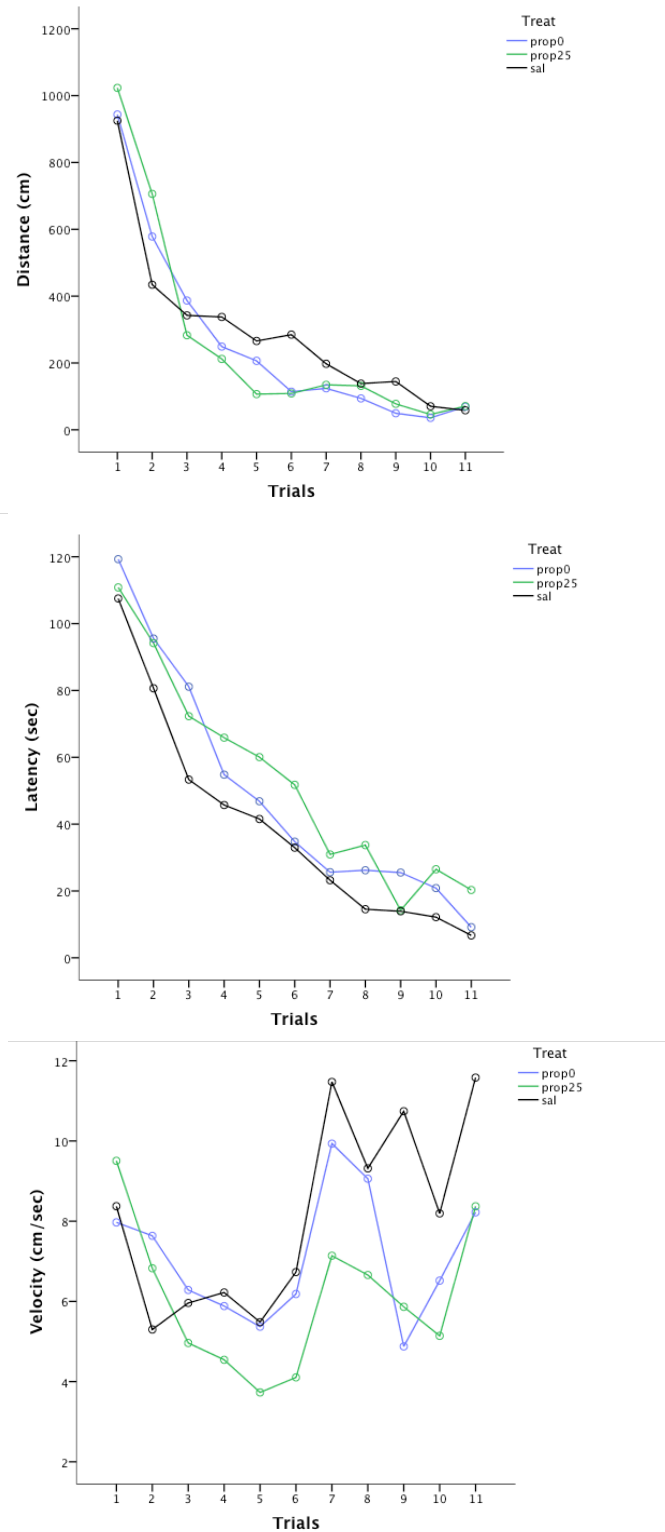


Figure 6. Average distance, latency of escape, and velocity by propranolol treatment condition over the eleven pre-treatment trials. Trial 11 was the reactivation trial.

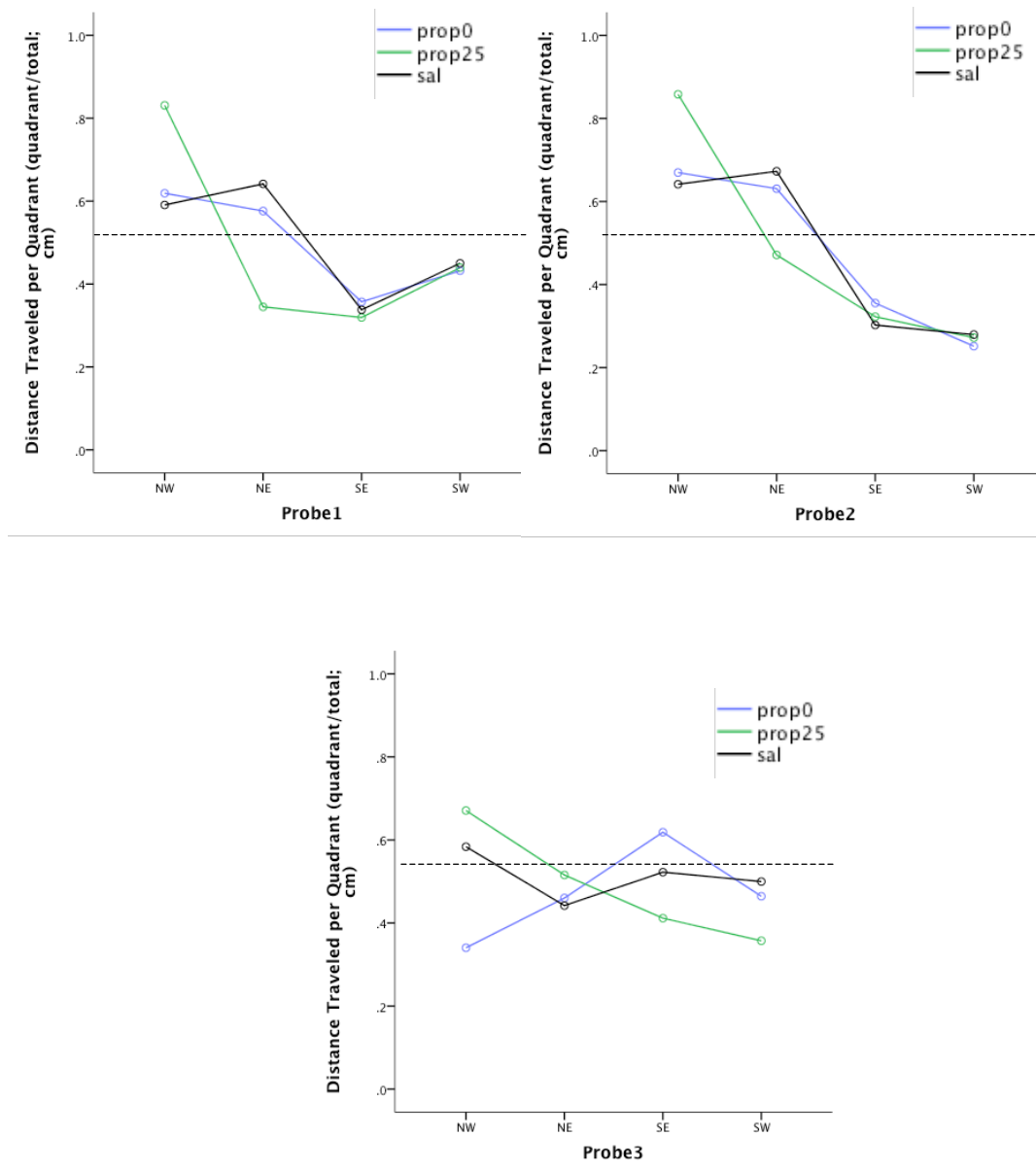


Figure 7. Propranolol Pre-treatment Probes. Distance traveled within each quadrant of the escape maze for each of the three pre-treatment probes. The dashed line indicates 25% chance (0.523).

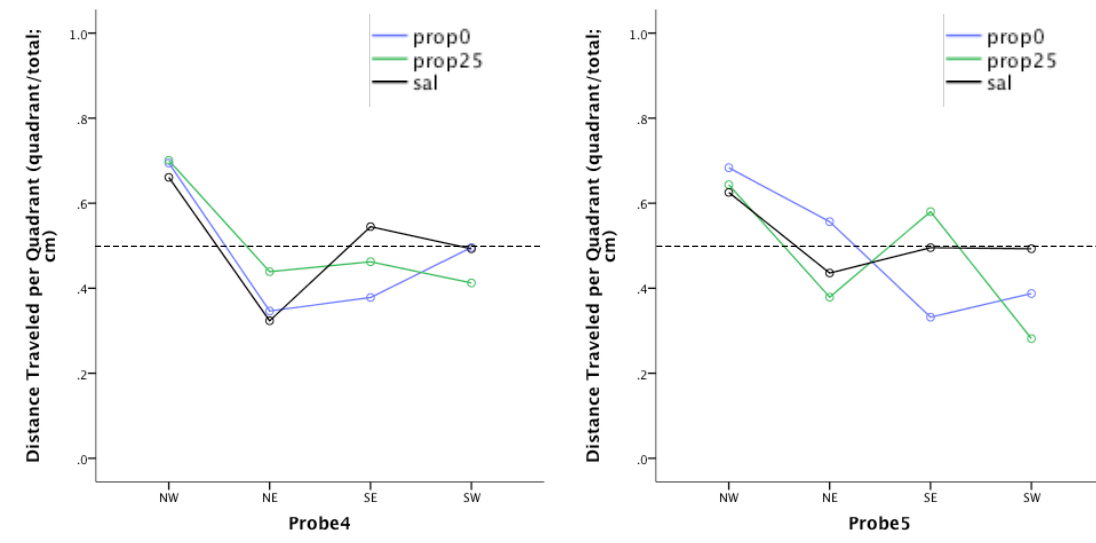


Figure 8. Propranolol Post-treatment Probes. Distance traveled within each quadrant of the escape maze for each of the two post-treatment probes. The dashed line indicates 25% chance (0.523).

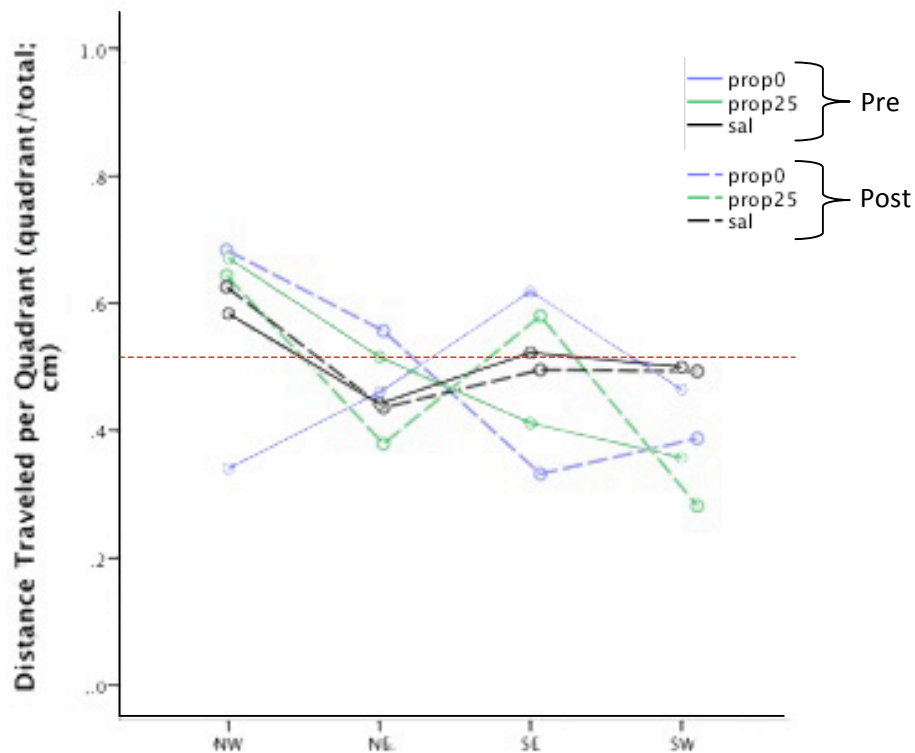


Figure 9. Propranolol Pre- to Post-treatment Probe Comparison (Probe 3 to Probe 5).

Distance traveled within each quadrant of the escape maze for Probe 3 (a pre-treatment aviary rotation probe) and Probe 5 (a post-treatment probe conducted consistent with Probe 3). Solid lines on the graph indicate Probe 3 values while dashed lines indicate Probe 5 values. The red dashed line indicates 25% chance (0.523).

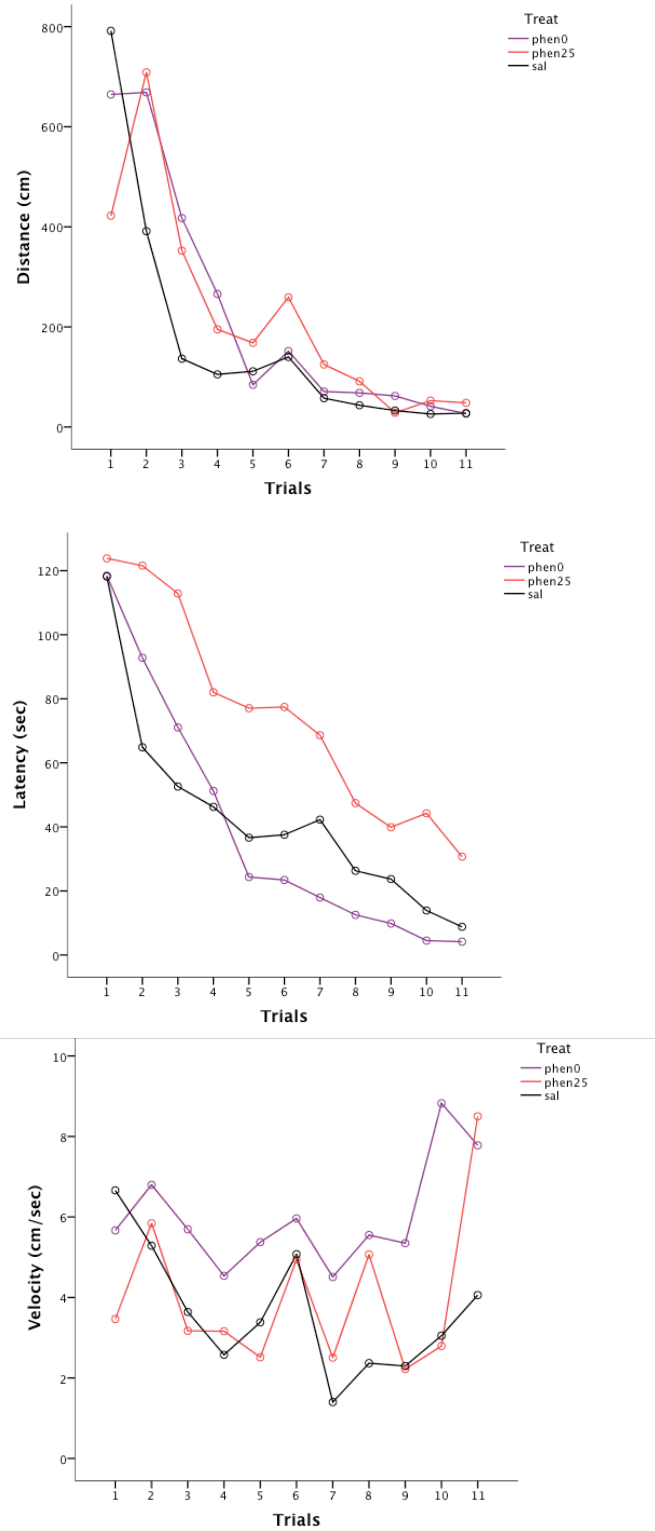


Figure 10. Average distance, latency of escape, and velocity by phentolamine treatment condition over the eleven pre-treatment trials. Trial 11 was the reactivation trial.

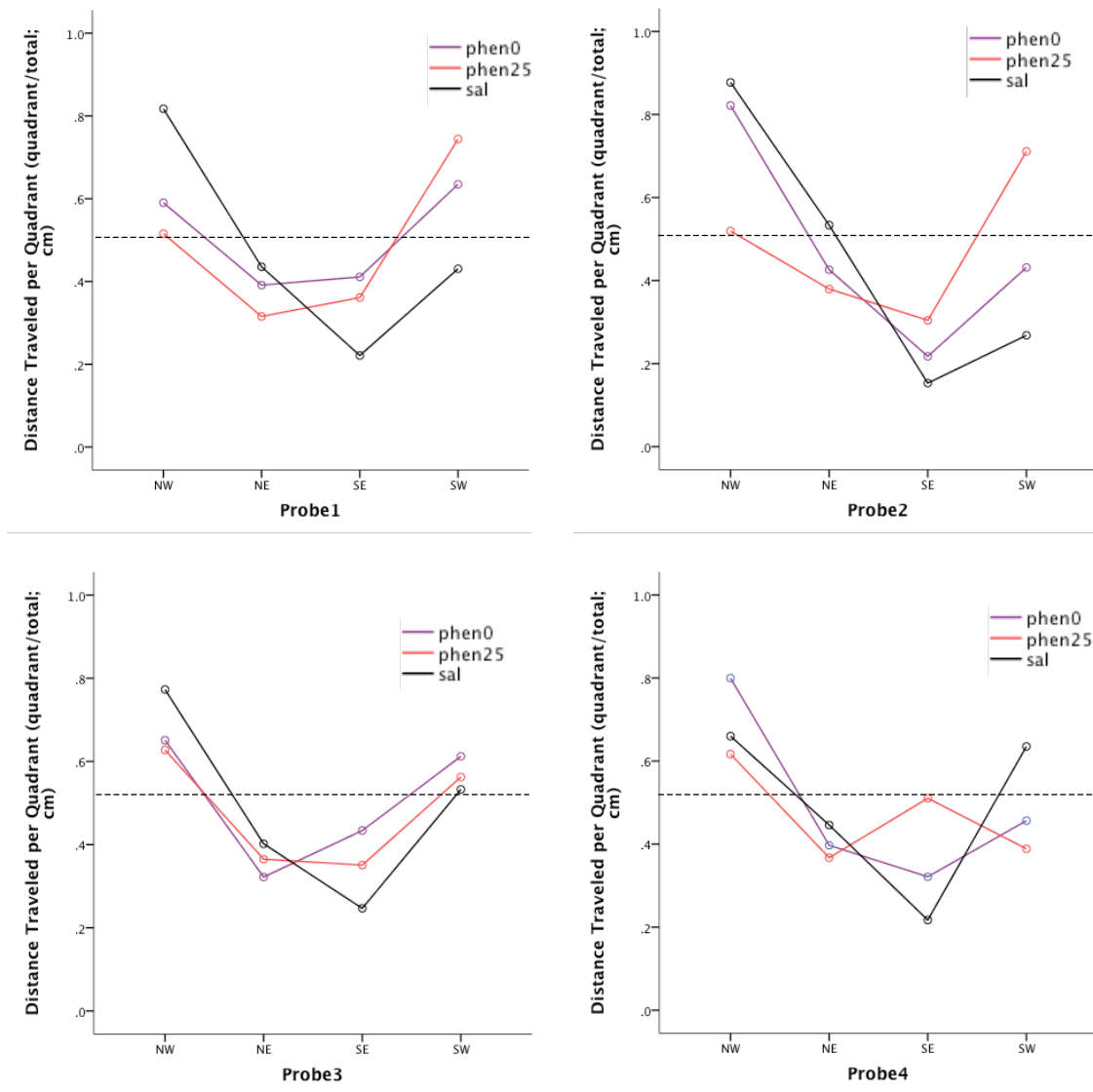


Figure 11. Phentolamine Pre-treatment Probes. Distance traveled within each quadrant of the escape maze for each of the four pre-treatment probes. The dashed line indicates 25% chance (0.523).

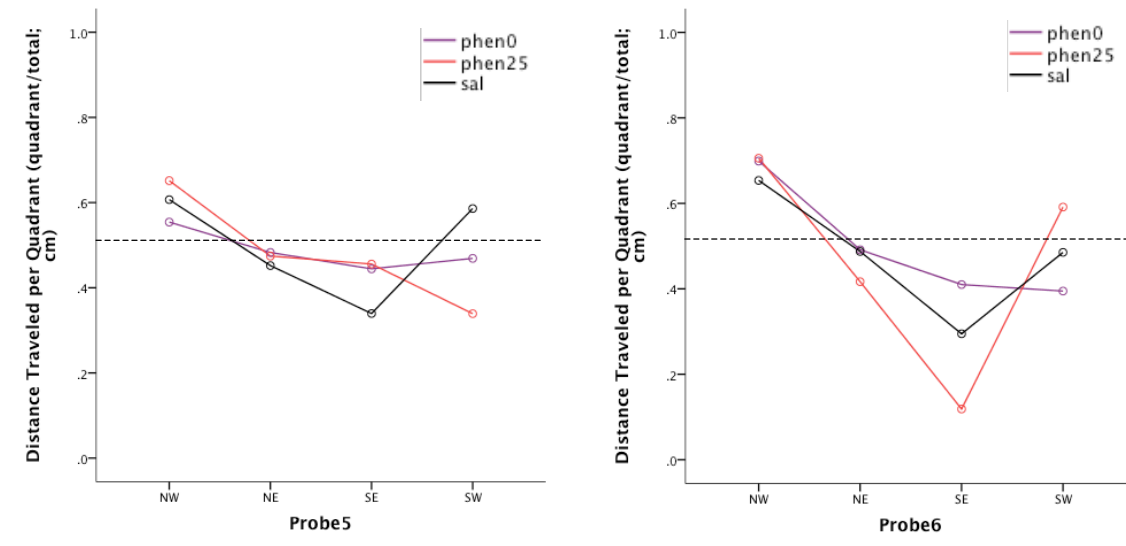


Figure 12. Phentolamine post-treatment probes. Distance traveled within each quadrant of the escape maze for each of the two post-treatment probes. The dashed line indicates 25% chance (0.523).

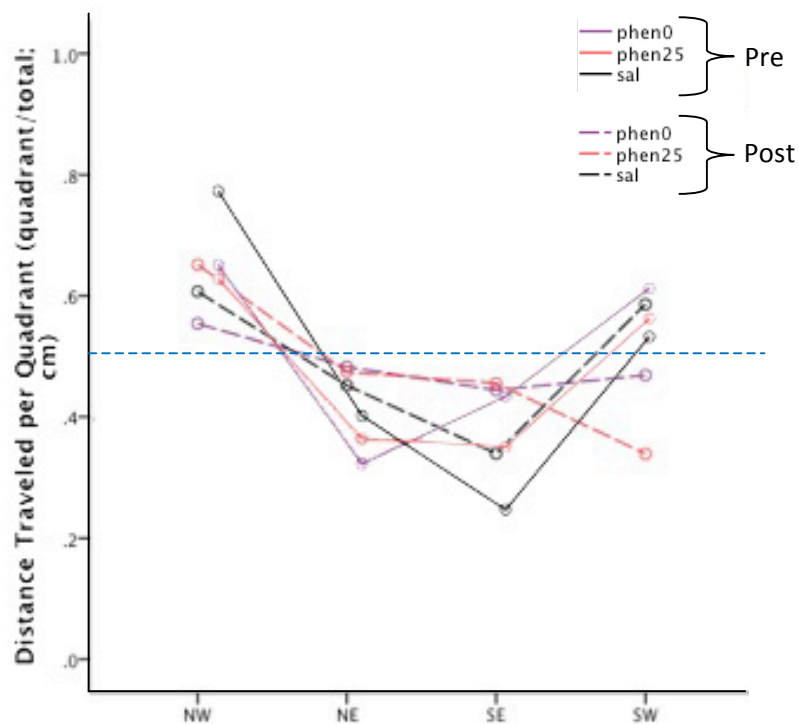


Figure 13. Phentolamine pre to post-treatment probe comparison (Probe 3 to Probe 5). Distance traveled within each quadrant of the escape maze for Probe 3 (a pre-treatment probe where only the escape hole was removed and all other variables remained constant) and Probe 5 (a post-treatment probe conducted consistent with Probe 3). Solid lines on the graph indicate Probe 3 values while dashed lines indicate Probe 5 values. The blue dashed line indicates 25% chance (0.523).

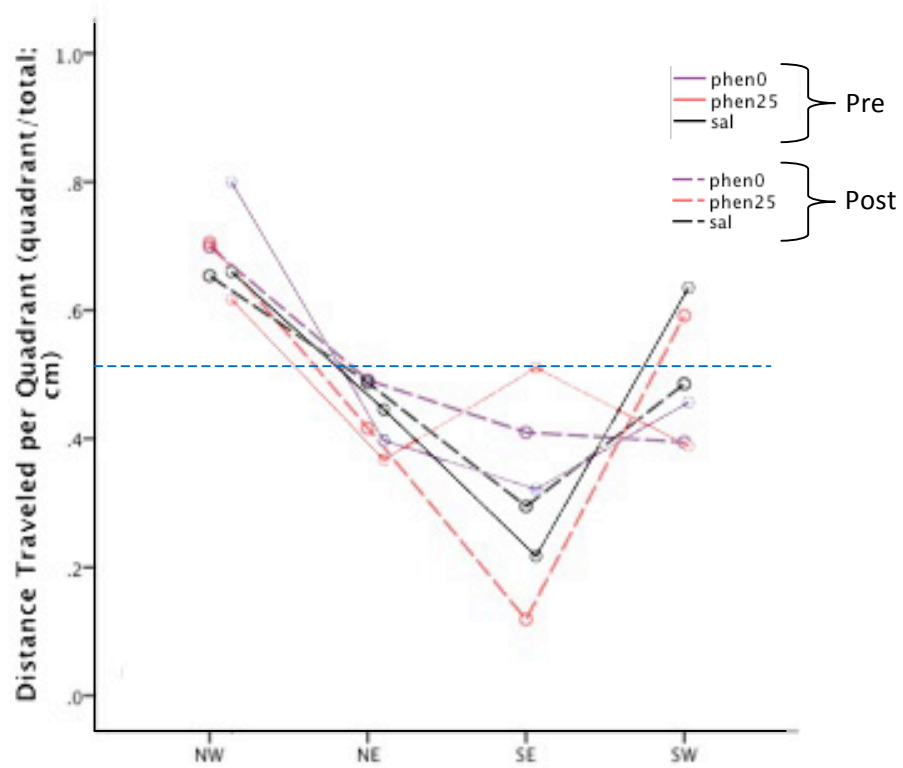


Fig. 14. Phentolamine Pre- to Post-treatment Probe Comparison (Probe 4 to Probe 6).

Distance traveled within each quadrant of the escape maze for Probe 4 (a pre-treatment proximal cue rotation probe) and Probe 6 (a post-treatment probe conducted consistent with Probe 4). Solid lines on the graph indicate Probe 4 values while dashed lines indicate Probe 6 values. The blue dashed line indicates 25% chance (0.523).